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Unconventional, adenosine-producing suppressor T cells induced by dendritic cells exposed to BPZE1 pertussis vaccine

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ABSTRACT

BPZE1 is a live attenuated pertussis vaccine that successfully completed a phase 1 safety trial. This article describes the induction of unconventional suppressor T cells-producing ADO by MDDCs exposed to BPZE1 (BPZE1-DC) through distinct ectoenzymatic pathways that limit the damaging effect of inflammation. BPZE1-DC induces $CD4^+$ and $CD8^+$ T lymphocytes to express 2 sets of ectoenzymes generating ADO: one set is part of the conventional CD39/CD73 pathway, which uses ATP as substrate, whereas the other is part of the CD38/CD203a/CD73 pathway and metabolizes NAD^+. The contribution of the ADO-generating ectoenzymes in the regulatory response was shown by 1) selective inhibition of the enzymatic activities of CD39, CD73, and CD38; 2) the ability of suppressor T cells to convert exogenously added ATP and NAD^+ to ADO; and 3) a positive correlation between ectoenzyme expression, ADO levels, and suppression abilities. Thus, T lymphocytes activated by BPZE1-DC shift to a suppressor stage, through the expression of ectoenzyme networks, are able to convert extracellular nucleotides into ADO, which may explain the potent anti-inflammatory properties of BPZE1 observed in several murine models. *J. Leukoc. Biol.* 98: 000–000; 2015.

Introduction

The immune system is constantly challenged by microbial pathogens that invade, survive, and replicate in the mammalian host to establish persistent infection. An excess activation of innate and adaptive immune responses may then occur, which is potentially detrimental for the host. Tregs include distinct populations that are all capable of modulating excessive immune activation and its pathologic consequences. The expression of the canonical transcription factor Foxp3 leads to the identification of 2 $Foxp3^+$ subsets. The first comprises Tregs of thymic origin, which are crucial in maintaining immune tolerance. The second includes Tregs generated in the periphery, after antigenic stimulation [1]. $Foxp3^+$ Treg cells are flanked by other subsets reported as $Foxp3^-$. These include type 1 regulatory T cells, prevalently secreting IL-10; Th3 Tregs, expressing TGF-β; and $CD8^+$ Tregs [2–6]. A functional distinction can be made between Treg subsets based on their modes of action, which include cytolysis of effector T cells, secretion of inhibitory cytokines, inhibition of DC maturation, and metabolic disruption [7, 8].

Coexpression of the ectonucleotidases CD39 and CD73 is one of the key mechanisms of immunosuppression through metabolic disruption. When cell damage occurs, for instance during tissue injury or inflammation, ATP is released into the extracellular space. Although extracellular ATP acts on several immune cells to promote inflammation, the ATP metabolite ADO is endowed with anti-inflammatory properties [9, 10]. CD39 hydrolyzes extracellular ATP into ADP and then AMP, which, in turn, is rapidly degraded to ADO by CD73, thereby controlling this pathway of immune modulation [11].

Bordetella pertussis is the causative agent of pertussis (whooping cough), a disease that still represents a major threat to public health [12, 13]. The immunity conferred by acellular pertussis vaccines, introduced in the last decade of the past century, wanes over time [14–18]. High vaccine coverage in industrialized countries has not prevented periodic outbreaks affecting people all ages, including adolescents and adults [15, 19]. The consequence is the formation of a reservoir for transmission to

Abbreviations: %A = percentage of the total peak area, ADO = adenosine, APCP = α,β-methylene ADP, BPZE1-DC = BPZE1-treated monocyte-derived dendritic cells, DC = dendritic cell, DC-none = unstimulated dendritic cell, DYP = dipyridamole, EHNA = erythro-9-(2-hydroxy-3-nonyl)-adenine, Foxp3 = forkhead box P3, MDDC = human monocyte-derived dendritic cell, POM-1 = sodium polyoxotungstate, Tregs = regulatory T cells

The online version of this paper, found at www.jleukbio.org, *includes supplemental information.*

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infants, who show the highest risks of developing severe pertussis and death [15, 20].

BPZE1 is a live attenuated *B. pertussis* strain developed as a vaccine candidate against pertussis, which has successfully completed a phase 1 safety trial [21–24]. BPZE1 and its virulent, parental strain BPSM are both able to promote the phenotypic maturation of MDDC. BPZE1- and BPSM-treated MDDCs drive a mixed Th1/Th17 polarization and, at the same time, acquire the ability to expand T cells with suppressor activity [25, 26]. These suppressor cells show unconventional characteristics because they do not act through IL-10 or TGF-β nor do they express increased levels of Foxp3 [25].

The present study describes the events leading to suppression. Our observations indicate that T cells primed by BPZE1-treated MDDCs (BPZE1-DC) acquire the ability to generate ADO as the product of 2 distinct, purine-sensing ectoenzyme pathways, namely the canonical CD39/CD73 and the recently described CD38/CD203a/CD73 networks.

MATERIALS AND METHODS

Ethic statement

This study was conducted according to the principles of the Declaration of Helsinki and the Code of Ethics of the Istituto Superiore di Sanità. Peripheral blood was collected from healthy blood donors at the Centro Trasfusionale Policlinico Umberto I, University La Sapienza blood bank (Rome, Italy; courtesy of Dr. Girelli). The blood samples were handed over anonymously. None of the authors were involved in collecting the blood samples or had access to patient identifying information. All blood donors provided written informed consent for the collection of samples and subsequent analysis. Blood samples were processed anonymously; the materials once used for the experiments were then destroyed, and genetic research or interventions that included genome analysis were not included in the research protocol. This study was conducted within project Child-INNOVAC, in compliance with European Commission FP7 ethical rules.

Chemicals

NAD^+, AMP, ADP, ATP, potassium dihydrogen phosphate (KH_2PO_4) and acetonitrile (HPLC-grade reagent) were all from Sigma-Aldrich (St. Louis, MO, USA) as well as APCP (CD73 ecto-5′-nucleotidase inhibitor), POM-1 (CD39 NTPDase inhibitor), kuromanin (CD38 ADP-ribosylation cyclase inhibitor), EHNA (adenosine deaminase inhibitor), levamisole (alkaline phosphatase inhibitor), and DYP (nucleoside transporter inhibitor). All other chemical reagents used were of analytical grade.

Bacterial strains and growth conditions

The BPZE1 strain used in this study is derived from *B. pertussis* Tohama I and has been described previously [21, 25]. Bacteria were grown on charcoal agar plates supplemented with 10% sheep blood (Oxoid, Basingstoke, United Kingdom) for 48 h at 37°C. Cells were then collected and resuspended in 2 ml PBS, and the concentration was estimated by measuring the optical density at 600 nm. The suspensions were adjusted to a final concentration of 10^9 CFU/ml. The bacterial concentration was then checked retrospectively by CFU evaluation of the final bacteria suspension.

Purification and culture of MDDC

Human monocytes were purified, as described [25], from peripheral blood and cultured in RPMI 1640 medium (GIBCO Invitrogen, Paisley, United Kingdom), supplemented with heat-inactivated 10% LPS-screened FCS, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, all from HyClone Laboratories (South Logan, UT, USA) and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich) (hereafter defined as *complete medium*) in the presence of GM-CSF (25 ng/ml) and IL-4 (25 ng/ml), both from R&D Systems (Minneapolis, MN, USA). After 6 d, immature MDDCs were washed and analyzed by cytofluorometry for the expression of surface markers CD1a, CD14, CD83, and CD38 with a panel of fluorochrome-conjugated mAbs (BD Biosciences, San Jose, CA, USA). MDDCs were used in the experiments if >80% CD1a and <10% CD14.

MDDCs (10^6 cell/ml) were resuspended in complete medium, without penicillin and streptomycin, and treated with BPZE1, as described elsewhere [25]. After 2 h, cells were extensively washed in the presence of polymyxin B (5 μg/ml) to kill extracellular bacteria and incubated at 37°C, 5% CO_2 for 48 h in complete medium.

Suppression assay

$CD45RA^+$-naïve T cells were purified from peripheral blood mononuclear cells, $CD3^+$ T cells were initially purified by negative sorting with magnetic beads (Pan T-cell Kit, Miltenyi Biotec, Bergisch Gladbach, Germany). Naïve T cells were successively purified from bulk by negative sorting with anti-CD45RO-conjugated magnetic beads (Miltenyi Biotec). Purified naïve T cells were exposed to allogeneic MDDC treated with BPZE1 (MDDC:T cell ratio 1:10 primary MLR). In some experiments, primary MLR was performed with purified $CD4^+$ and $CD8^+$ naïve T cells obtained by negative sorting with anti-CD4- or anti-CD8-conjugated magnetic beads (Miltenyi Biotec). At day 6, T cells from the primary MLR were recovered and cultured at different ratios with syngeneic T cells (5×10^5) in the presence of allogeneic LPS-matured MDDCs (0.5×10^5) in 48-well cell culture plates (secondary MLR). Cell proliferation in MLR was measured by BrdU incorporation and evaluated by direct immunofluorescence with a FITC-conjugated anti-BrdU mAb (BD Biosciences). Briefly, BrdU (BD Biosciences) was added to MDDC and T cell MLR at 3 μg/ml final concentration on day 3 of culture. Cells, collected on day 6, were fixed in 0.5% paraformaldehyde, permeabilized, and stained for intracellular BrdU by direct immunofluorescence with a FITC-conjugated anti-BrdU mAb (BD Biosciences). Cells were examined by flow cytometry, and T cell proliferation was evaluated. Proliferation index was calculated for each independent experiment as the percentage of proliferation of T responder cells in the secondary MLR compared with proliferation in the absence of T suppressor cells.

In some experiments, secondary MLRs were performed in the presence of 1 μM POM-1 (inhibitor of CD39), 50 μM APCP (inhibitor of CD73), or 1 μM kuromanin, (inhibitor of CD38).

T cell immunophenotypic analysis

Cells were washed and resuspended in PBS containing 3% FBS and 0.09% NaN_3, then incubated with a panel of fluorochrome-conjugated mAbs: namely anti-CD3-APCH7, anti-CD4, PerCPCy5, anti-CD39-APC, anti-CD73-PECy7, anti-CD38-PE (BD Biosciences), and anti-CD203a-FITC (PC-1, clone 3E8; J. Goding, Institution, City, Country). Isotype-matched mAbs were used as negative controls. Cells were analyzed with a FACSCanto System (BD Biosciences). Analysis was performed with the FlowJo vX.0.7 software (TreeStar Inc, Ashland, OR, USA) and the Kaluza Analysis 1.3 software (Beckman Coulter, Miami, FL, USA). The gating strategy is shown in Supplemental Figure 1. AQ:2

ADO generation assay and HPLC analysis

ADO production was evaluated using 2×10^6/ml $CD3^+$ T cells maintained for 30 min at 37°C in AIM V serum-free medium (Invitrogen) containing NAD^+, ATP, or AMP (100 μM) in the presence of EHNA, levamisole, and DYP inhibitors. After centrifugation (700 *g*; 5 min at 4°C), supernatants were collected and diluted 1:2 with acetonitrile (Sigma-Aldrich) at 4°C to stabilize

AQ:3 the ADO. Supernatants were evaporated by SpeedVac, reconstituted in mobile-phase buffer, and assayed by HPLC.

Chromatographic analysis was performed with an HPLC system (Beckman Gold 126/166NM, Beckman Coulter, Milan, Italy) equipped with a reverse-phase column (Phenomenex C18, 5 μm; 250 × 4.5 mm). Separation of nucleotides and nucleosides was performed using a mobile-phase buffer (0.125 M citric acid and 0.025 M KH_2PO_4), pH 5.1, with 8% acetonitrile over 10 min at a flow rate of 0.8 ml/min. UV absorption spectra were measured at 254 nm. HPLC-grade standards used to calibrate the signals were dissolved in AIM V, pH 7.4, 0.2 μm sterile-filtered, and injected in a buffer volume of 20 μl. The retention times (in minutes) of standards were 2.15 for AMP, 2.8 for NAD^+, 3.2 for ADP ribosylation, 6.87 for nicotine, and 5.56 for ADO. Peak integration was performed using 32 Karat software (Beckman Coulter). The qualitative identity of the HPLC peaks was confirmed by comigration and absorbance spectra of known reference standards using a retention time window of ±5%. Quantitative measurements of relative ADO production were expressed as a %A [27].

Statistical analysis

Statistical descriptive analyses were carried out using the Prism statistical package (Graph Pad, La Jolla, CA, USA). Differences among mean values were assessed using the 2-tailed Student's *t* test and were statistically significant for *P* values <0.05.

RESULTS

BPZE1-DCs confer suppressor activity to $CD4^+$ and $CD8^+$ T subsets by a coordinated increase of CD39 and CD73 expression

The first set of experiments was designed to see whether the suppressor activity acquired by T cells after coculture with BPZE1-DC was attributable to $CD4^+$ or $CD8^+$ subsets. Therefore, we performed an MLR by coculturing DC 1) with heterologous total, naïve $CD3^+$ T cells; 2) with naïve $CD4^+$ T cells; or 3) with naïve $CD8^+$ T cells purified by magnetic bead selection from a single blood donor. After 6 d, T lymphocytes from the primary MLRs were harvested and added in increasing doses to secondary MLRs to test their ability to suppress the proliferation of autologous responder T cells. Cell proliferation in the secondary MLR was evaluated by measuring BrdU incorporation. The results indicate that the suppressor activity is conferred by BPZE1-DC to total $CD3^+$ T cells, as expected [25], as well as to the $CD4^+$ and the $CD8^+$ subsets, whose suppressor activity was comparable because the average proliferation index of responder T cells, observed when the Tregs:T responder ratio was 1:1, was 41.6% for purified CD4 T cells, and 46.2% for purified CD8 T cells (**Fig. 1**). $CD3^+$ and $CD4^+$ T lymphocytes acquired a certain degree of suppressor activity after coculture with DC-none (Fig. 1A and B), which likely explains why the presence of BPZE1 significantly enhanced the induction of suppressor activity of $CD8^+$, but not $CD4^+$, T cells, as compared with control DCs.

Both $CD4^+$ and $CD8^+$ T cells enriched in the $CD39^+CD73^+$ subpopulation are reported to suppress T cell effector functions in a mouse melanoma model [28]. Furthermore, CD39 expressed by $CD8^+$ T cells mediates suppression activities in different settings [29, 30]. These notions prompted us to investigate whether suppression mediated by BPZE1-DC-primed T cells is related to the generation of ADO. Therefore, the induction of surface CD39 and CD73 expression in the $CD4^+$ and the $CD8^+$ subsets was examined after the primary DC/naïve

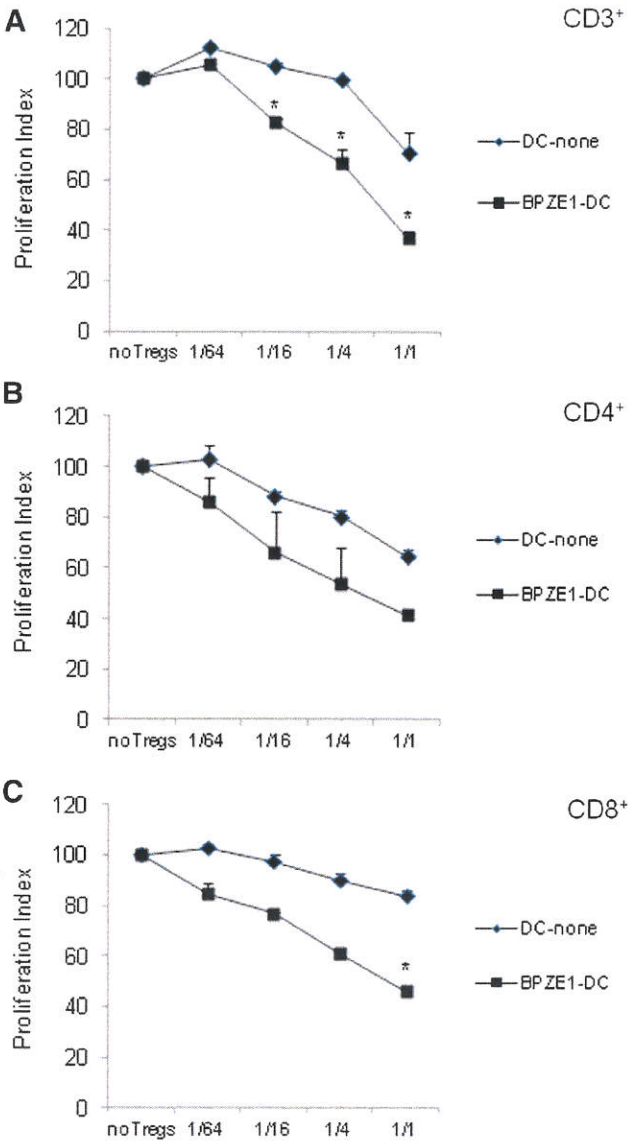

Figure 1. BPZE1-DC-primed $CD4^+$ and $CD8^+$ T cells are endowed with suppression activity. MDDC either untreated (DC-none) or challenged with BPZE1 (BPZE1-DC) for 48 h were cocultured with purified allogeneic (A) $CD3^+CD45RA^+$ naïve T cells, (B) $CD4^+CD45RA^+$ naïve T cells, or (C) $CD8^+CD45RA^+$ naïve T cells from the same donor (1:10 ratio) to generate suppressor T cells (Tregs). On day 6, T cells were collected and cocultured at different ratios with syngeneic T cells (5×10^5) in the presence of allogeneic LPS-matured MDDC (5×10^4). Proliferation of T cells in the secondary MLR was assessed by BrdU incorporation and expressed as a proliferation index, determined as the percentage of BrdU incorporation compared with incorporation in the absence of Tregs for each independent experiment. Results are reported as the means ± SE of 3 independent experiments performed with MDDC and T cells obtained from different donors. Means ± SE of $BrdU^+$ $CD3^+$ T cells in the absence of Tregs: 54.2 ± 6.1; $CD4^+$ T cells: 52.8 ± 4.1; $CD8^+$ T cells: 49.5 ± 7.3. *$P < 0.05$ vs. DC-none.

T-cell MLR. **Figure 2** shows that only a few naïve T cells primed by DC-none stained double positive in both T cell subsets. In contrast, the percentage of double positive $CD39^+CD73^+$ cells

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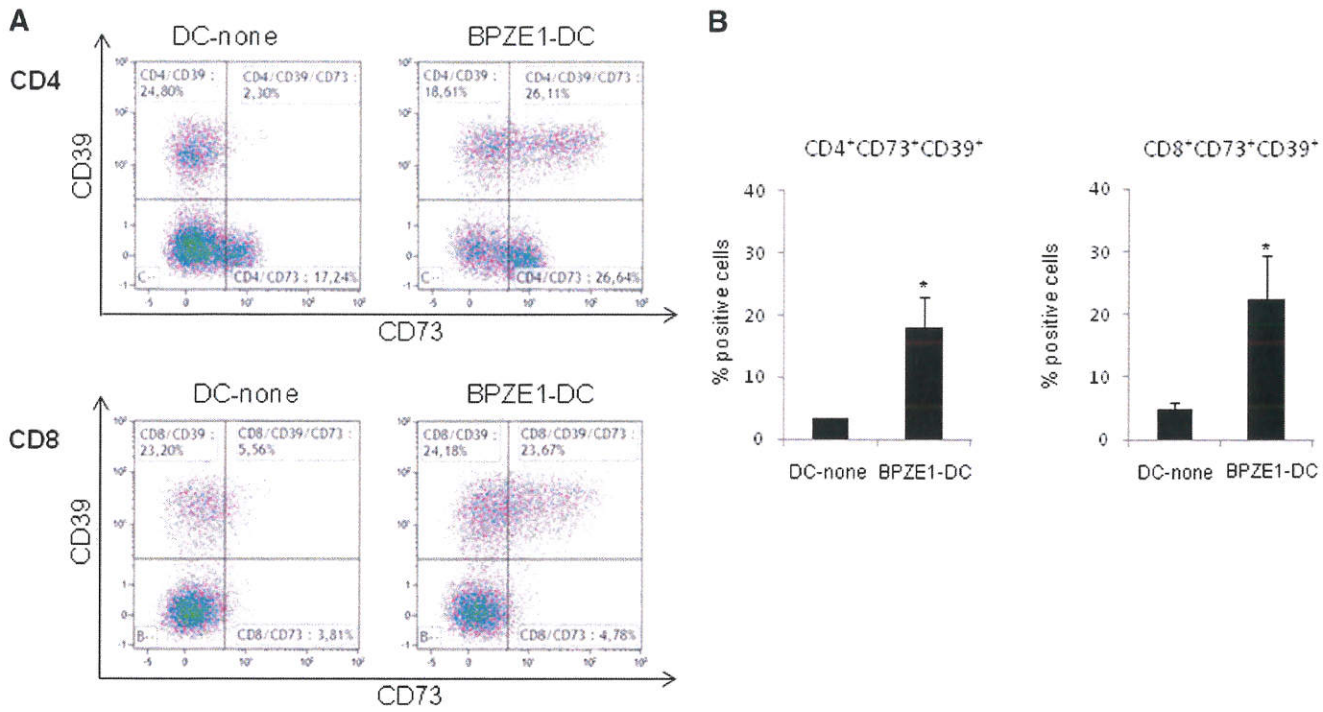

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Figure 2. BPZE1-DC-primed CD4$^+$ and CD8$^+$ T cells are enriched in a double-positive CD39$^+$CD73$^+$ subset. MDDC, either untreated (DC-none) or challenged with BPZE1 (BPZE1-DC) for 48 h, were cocultured with purified allogeneic CD3$^+$CD45RA$^+$ naïve T cells (1:10 ratio). On day 6, T cells were harvested and stained for the expression of surface markers. (A) Representative dot-plot showing CD39 and CD73 expression in CD4$^+$ and CD8$^+$ subsets. (B) Percentages of positive cells are reported as the means ± SE of 8 independent experiments performed with MDDC and T cells obtained from different donors. *$P < 0.05$ vs. DC-none.

greatly increased in T cells primed by BPZE1-DC, both in the CD4$^+$ and in the CD8$^+$ subsets, reaching statistical significance when compared with DC-none-primed T cells.

BPZE1-DCs induce CD4$^+$ and CD8$^+$ T cell subsets that simultaneously express ATP- and NAD$^+$-converting ectoenzymes

CD73 is an ADO-producing surface molecule shared by the conventional CD39/CD73 pathway and by a NAD$^+$-consuming ectoenzyme cascade. The latter includes the ectoenzymes CD38 and CD203a [27, 31]. Extracellular NAD$^+$ and ATP both serve as danger signals meant to alert the immune system [32]. In this case, extracellular NAD$^+$ is degraded by CD38 to ADP-ribose, and ADP-ribose is then converted into AMP by CD203a and into ADO by CD73 [27].

We investigated whether the coculture of naïve T cells with BPZE1-DC induces CD38 and CD203a, along with CD73. Phenotypic analysis indicated the existence of a triple-positive CD38$^+$CD203a$^+$CD73$^+$ population, along with the double-positive CD39$^+$CD73$^+$ subset induced by BPZE1-DC in the CD4$^+$ and in the CD8$^+$ T cell subsets. A quadruple-positive CD39$^+$CD38$^+$CD203a$^+$CD73$^+$ subpopulation was also induced in both the CD4$^+$ and the CD8$^+$ T cells (**Fig. 3** and Supplemental Fig. 1). Thus, the CD4$^+$ and CD8$^+$ T subsets have the potential for consuming extracellular ATP and NAD$^+$ for the production of ADO. Statistical significance was reached after comparison with the differential expression of the ectoenzymes in T lymphocytes after coculture with untreated DC, both in the CD4$^+$ and the CD8$^+$ subsets.

Selective inhibition of ADO-generating ectoenzymes reduces the suppressive functions of the T lymphocytes induced by BPZE1-DC

Phenotypic analysis of T lymphocytes induced by BPZE1-DC suggests that their suppressor activities are mediated by ADO generated by the distinct ATP- and NAD$^+$-consuming ectoenzymes, expressed by both the CD4$^+$ and CD8$^+$ subsets. This hypothesis was addressed by carrying out suppression assays in the presence of selective ectoenzymatic inhibitors. To this purpose, APCP (an inhibitor of CD73), POM-1 (an inhibitor of CD39), or kuromanin (an inhibitor of CD38) were added to a secondary MLR to assess any reduction in suppressor potential. As shown in **Fig. 4**, the inhibitors selected significantly limited the suppressor activity of T cells primed with BPZE1-DC. The lowest degree of inhibition was observed when kuromanin was used. The scarce degree of suppression exerted by T cells primed with DC-none was not affected by ectoenzymatic inhibitors. These findings indicate that ADO-generating ectoenzymes have key roles in the regulatory response mediated by T cells cocultured with BPZE1-DC.

T cells primed by BPZE1-DC are able to degrade ATP, NAD$^+$, and AMP into ADO

To examine whether ADO-mediated suppression occurs through the CD39/CD73 and/or the CD38/CD203a/CD73 pathways, respectively, and whether extracellular ATP or NAD$^+$ is metabolized, naïve T cells were primed with BPZE1-DC for 6 d and then harvested and incubated with ATP, NAD$^+$, or AMP, the

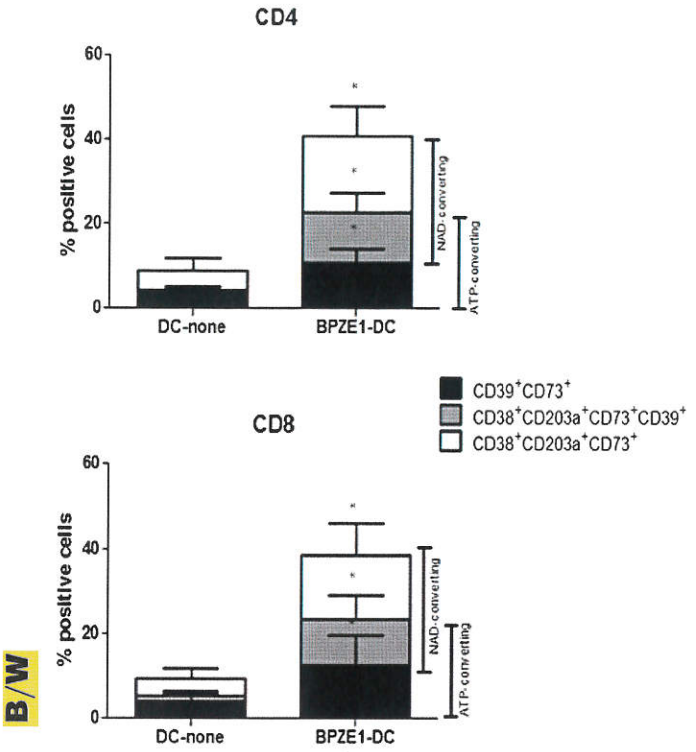

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Figure 3. BPZE1-DC-primed CD4$^+$ and CD8$^+$ T cells express ADO-converting ectoenzymatic pathways. MDDC either untreated (DC-none) or challenged with BPZE1 (BPZE1-DC) for 48 h, were cocultured with purified allogeneic CD3$^+$CD45RA$^+$ naïve T cells (1:10 ratio) to generate suppressor T cells. On day 6, T cells were harvested and stained for the expression of surface markers. Data are expressed as the percentage of positive cells (means ± SE) from 7 independent experiments. *$P < 0.05$ vs. DC-none.

latter being converted to ADO by both enzymatic pathways [27]. ADO levels in cell culture supernatants were afterward quantified by HPLC. As shown in **Fig. 5**, both the ATP-degrading and NAD$^+$-degrading pathways are active in the same population of T cells primed by BPZE1-DC, as the addition of either exogenous substrates produced increased ADO levels. However, the CD39/CD73 pathway appeared to be more efficient than the CD38/CD203a/CD73 pathway was. Differences in ADO production were statistically significant when compared with DC-none-primed T cells.

The levels of ectoenzyme surface expression, suppression activity, and ADO production correlate positively

The relation between the induction of ADO-generating ectoenzymes on T cells by BPZE1-DC and suppression activity was analyzed by comparing, for each independent experiment, the percentage of ectoenzyme-expressing T cells after the primary MLR with the maximal suppression achieved in the secondary MLR. As shown in **Fig. 6A**, there is a statistically significant positive correlation between the percentage of CD39$^+$CD73$^+$ or CD38$^+$CD203a$^+$CD73$^+$ T lymphocytes expanded in the primary MLR and suppression activity.

Expression data were then compared with the functional ability of T cells to generate ADO when exposed to exogenous ATP or NAD$^+$ as substrates (Fig. 6B). A strong correlation was found between the percentage of CD39$^+$CD73$^+$ T cells expanded in the primary MLR and the capacity to generate ADO when incubated in vitro with exogenous ATP. Similarly, a positive correlation was also found between the percentage of CD38$^+$CD203a$^+$CD73$^+$ cells induced after the primary MLR and the capacity to convert NAD$^+$ into ADO. A significant correlation was

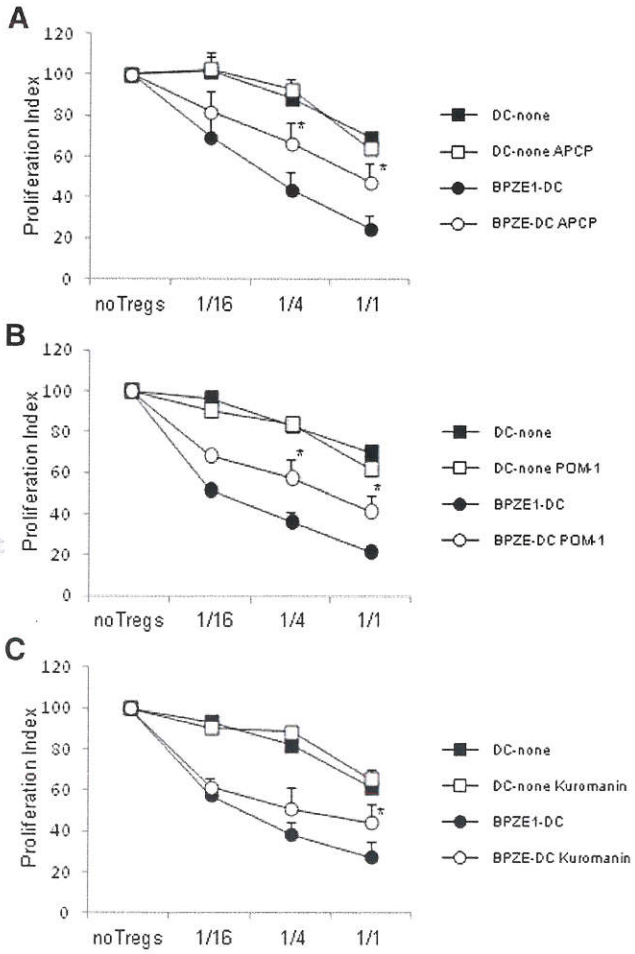

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Figure 4. Ectoenzyme inhibition limits the suppressor functions of BPZE1-DC primed T cells. MDDC either untreated (DC-none) or challenged with BPZE1 (BPZE1-DC) for 48 h, were cocultured with purified allogeneic CD3$^+$CD45RA$^+$ naïve T cells (1:10 ratio) to generate suppressor T cells (Tregs). On day 6, T suppressor cells were collected and cocultured at different ratios with syngeneic T cells (5×10^5) in the presence of allogeneic LPS-matured MDDC (5×10^4) and specific enzymatic inhibitors of (A) CD73 (APCP), (B) CD39 (POM-1), and (C) CD38 (kuromanin) were added to the cultures. Proliferation of T cells was assessed by BrdU incorporation and expressed as a proliferation index, calculated as the percentage of proliferation of T responder cells in the secondary MLR compared with proliferation in the absence of Tregs for each independent experiment. Results are reported as means ± SE of 4 independent experiments performed with MDDC and T cells obtained from different donors. Means ± SE of BrdU$^+$ CD3$^+$ T cells in the absence of Tregs: 49.8 ± 3.1. *$P < 0.05$ vs. DC-BPZE1.

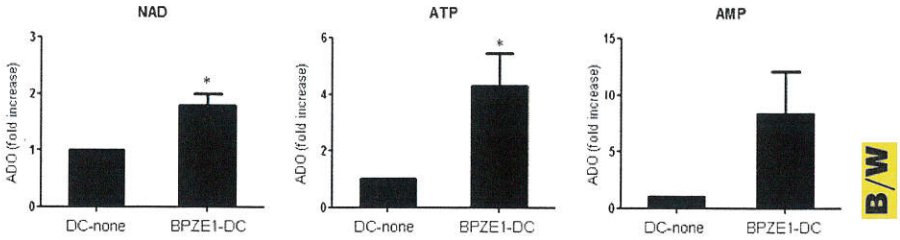

Figure 5. BPZE1-DC-primed T cells convert exogenous nucleotides to ADO. MDDC, either untreated (DC-none) or challenged with BPZE1 (BPZE1-DC) for 48 h were cocultured with purified allogeneic $CD3^+CD45RA^+$ naïve T cells (1:10 ratio) to generate suppressor T cells. On day 6, T cells were harvested and incubated with 0.1 mM NAD^+, 0.1 mM ATP, or 0.1 mM AMP. Generation of ADO was measured by HPLC. Quantitative measurements were inferred by comparing the peak area of the samples with the calibration curves for peak areas of each standard compound. Values are expressed as the fold increase in ADO production compared with DC-none-primed T cells and are the means ± SE of 4 independent experiments. The %A of NAD^+-incubated DC-none: 0.34 ± 0.06; %A of ATP-incubated DC-none: 3.42 ± 1.18; %A of AMP-incubated DC-none: 3.67 ± 1.52. $^*P < 0.05$ vs. DC-none.

also found between the capacity of T cells to generate ADO in vitro when exposed to exogenous ATP or NAD^+ as substrates and the percentage of suppression exerted in the secondary MLR (Fig. 6C).

DISCUSSION

The induction of a homeostatic response is crucial upon immunization or natural infection. Long-term immune activation and high levels of proinflammatory cytokines may lead to generalized damage in the body. As previously shown, BPZE1-DCs can promote proinflammatory Th1/Th17 responses along with regulatory T cell responses, which likely balance each other to fine-tune local homeostasis [25]. This observation is in line with findings derived from murine models, which revealed the anti-inflammatory properties of BPZE1 [33–35] and is supported by the results of a phase 1 trial [23, 24], which demonstrated the safety of BPZE1 in humans. The simultaneous expression of CD39 and CD73 has been reported for certain murine and human Tregs. The coordinated action of these ectonucleotidases leads ATP or ADP to be metabolized by CD39, and the metabolite is, in turn, degraded to extracellular ADO by CD73 [11]. The present study indicates that BPZE1-treated DCs promote the expansion from naïve T cells of $CD4^+$ and $CD8^+$ Tregs, which exert suppressor activities through their ability to generate ADO via the CD39/CD73 pathway. This contention is

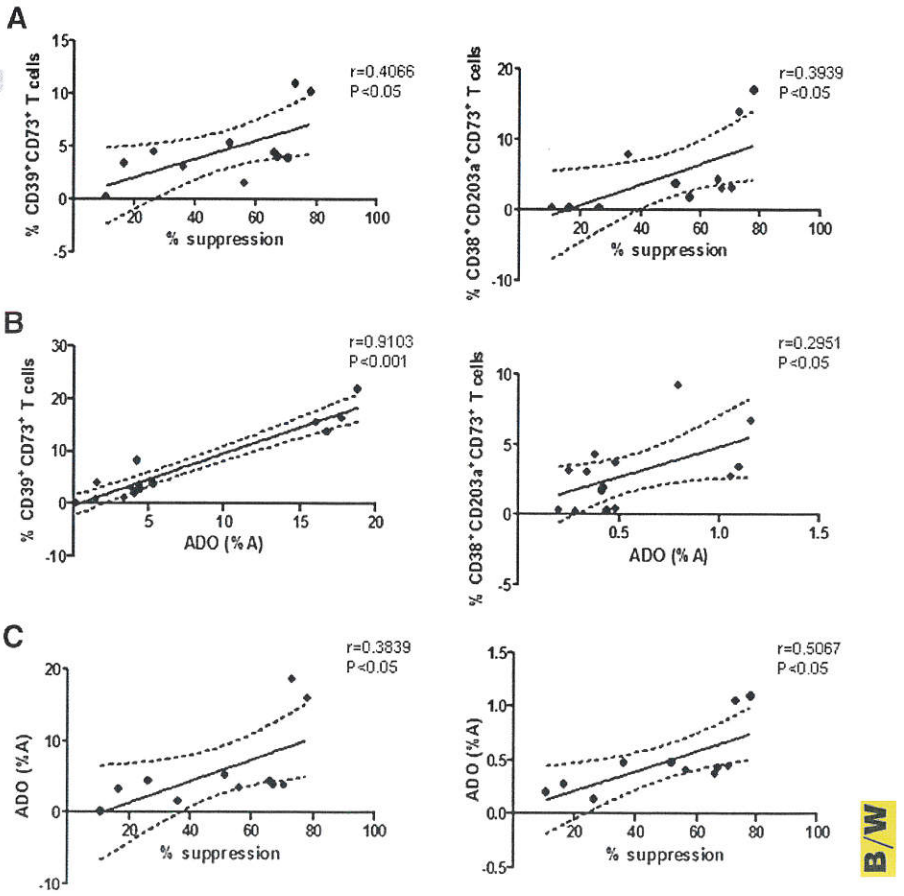

Figure 6. Ectoenzyme expression correlates with T cell suppression ability and ADO generation. (A) Linear regression correlating the percentage of $CD39^+CD73^+$ (left) or $CD38^+CD203a^+CD73^+$ (right) T cells with the percentage of suppression achieved when Tregs were added in a 1:1 ratio to a secondary MLR. *r* and *P* values are shown. (B) Linear regression correlating the percentage of $CD39^+CD73^+$ (left) or $CD38^+CD203a^+CD73^+$ (right) T cells with their capacity to generate ADO, expressed as %A, when incubated with ATP or NAD^+. *r* and *P* values are shown. (C) Linear regression correlating the capacity of T cells to generate ADO, expressed as %A, when incubated with ATP (left) or NAD^+ (right) with the percentage of suppression achieved when Tregs were added in a 1:1 ratio to a secondary MLR. *r* and *P* values are shown.

consistent with the observation that the suppressive potential of T cells primed by BPZE1-DC was significantly reduced when the activities of both ectoenzymes were metabolically blocked. These cells were also able to degrade the exogenously, in vitro, added ATP to ADO. Last, enrichment in the $CD39^+CD73^+$ cell subset finely paralleled the production of ADO from ATP.

The original observation of the present work shows that the functions exerted by BPZE1-DC-induced Tregs also rely on a set of distinct ectoenzymes, which metabolize NAD^+. CD38 and CD203a (a cell surface enzyme with nucleotidase pyrophosphatase/phosphodiesterase activity) and which have recently been proposed as leading an alternative route to production of extracellular ADO in the presence of CD73. At variance with the conventional network, this pathway consumes extracellular NAD^+: CD38 degrades NAD^+ into ADP ribosylation, which is then metabolized by CD203a into AMP, which provides CD73 with a substrate for the production of ADO [31]. Highlighted several years ago [36–38], this network has recently been characterized in detail in a human T lymphocyte model [27] and was shown to be active in the bone marrow myeloma niche [31]. T cells primed by BPZE1-DC not only up-regulate the expression of CD38, CD203a, and CD73 but also produce ADO when extracellular NAD^+ is added to the cultures. Furthermore, inhibition of the CD38 enzymatic activities reduces suppression.

The relevance of this pathway is confirmed by the identification of a small subset of T cells characterized by the quadruple expression of CD39, CD38, CD203a, and CD73, which express both the conventional and the alternative routes of ADO production. It is conceivable that these cells are characterized by high suppressive ability or by specific phenotypic profiles. Future studies are planned with the aim of sorting this subpopulation and addressing this aspect more specifically.

The present study demonstrates that the ectoenzymes armamentarium of $CD4^+$ and $CD8^+$ T cells is similar; further, phenotypic analysis is in line with the observation that both subsets are able to suppress the proliferation of responder T cells. These results are consistent with a number of apparently unrelated findings. For instance, high CD38 levels are found to correlate with the modulatory properties of $CD4^+$ T lymphocytes [39], and $CD8^+$ Tregs are reported to express nucleotide-degrading ectoenzymes [28–30, 40].

A further demonstration of the involvement of the ADO-generating ectoenzyme pathways in regulatory activity was provided by the positive correlation among expression levels of the components of the 2 ectoenzyme pathways on T cells: suppression activity and the generation of ADO produced after incubation with ATP or NAD^+ (Fig. 6). Thus, T lymphocytes induced by BPZE1-DCs are able to exploit ATP, NAD^+, or both to produce ADO, thereby being able to sense these key metabolites released into the extracellular compartment because of prolonged inflammation [32].

To understand whether the observed induction of ADO-generating suppressor T cells is specifically mediated by incubation of MDDC with BPZE1, in a few sets of experiments, naïve T cells were incubated with *Escherichia coli* LPS-primed MDDC, and their functions and phenotype were analyzed. We found that T cells cocultured with *E. coli* LPS-primed MDDC do not suppress responder T cell proliferation and do not up-regulate the expression of ADO-generating ectoenzymes (Supplemental Fig. 2).

Further experiments should be performed to ascertain the mechanisms through which BPZE1-DCs induce T cells with suppressor functions. It is possible to hypothesize that BPZE1 activates the expression of specific surface molecules, cytokines, or both, which induce ectoenzyme expression on the T cells. Alternatively, it could be envisaged that bacterial products may exert that function. We have shown in a previous study [41] that *B. pertussis* is unable to replicate in human MDDCs, and few hours after the infection, living bacteria are no longer inside the cells. However, it is not possible to exclude the possibility that, despite addition of polymyxin B and extensive washing, bacterial products persist in cell cultures attached to MDDC membranes and could then exert a direct effect on naïve T cells.

The recent findings from studies on the bone marrow niche of human myeloma reveal some previously unreported characteristics pertaining to the functionality of the NAD^+-consuming ectoenzyme network. The network may be continuous or discontinuous, implying that the components do not need to be expressed by the same cells, provided that happens in a closed system, which allows a free exchange of substrates and products [31]. Another pertinent observation is that the alternative pathway is more efficient in hypoxic conditions, typical of myeloma [31], and is also linked to *B. pertussis* infection [42].

In the context of pulmonary inflammation, either ATP or NAD^+ can be released [43, 44]. Not only substrates but also ectoenzymes are induced in inflamed airways. Indeed, a recent report indicates that a murine model of acute lung injury is characterized by the presence of T cell subsets expressing ectoenzymes that can consume ATP (CD39, CD73) or NAD^+ (CD38, CD203a, CD157, CD296) [45].

It is generally accepted that the induction of the CD39/CD73 system evolved as a feedback mechanism that is activated and expressed by conventional effector-like T cells to obtain a negative modulation of the immune response. Supporting this view, several reports [46–48] have described human $CD4^+$ $CD39^+$ T populations that are apparently $Foxp3^-$ but that exhibit a memory effector phenotype. Moreover, in vitro-generated Th17 cells express CD39 and CD73 at their surface and generate ADO, leading to suppression of $CD4^+$ and $CD8^+$ T effector functions [49]. Lastly, a mouse melanoma model demonstrated that $CD4^+Foxp3^-$ and $CD8^+$ T cells infiltrating melanoma lesions are enriched in a $CD39^+CD73^+$ population. The authors [28] proposed a mechanism of suppression of the immune effector cells that involves ADO produced by effector T cells. It is conceivable that a similar phenomenon may have occurred in the experimental system adopted in this study and that some of the antigen-stimulated T cells started to express ADO-generating ectoenzymes to curb overwhelming activation. In that case, BPZE1-DCs promote not only suppressor T cells but also activated T cells expressing IFN-γ and IL-17 [25].

In a previous study [25], we have shown that a small percentage of $CD4^+CD25^+Foxp3^+$ Tregs is similarly induced by either DC-none- or BPZE1-DC-primed T cells. Remarkably, DC-none-primed $CD4^+$ T cells acquired a limited degree of suppression (Fig. 1), which could be ascribed to classic $Foxp3^+$ Tregs.

In conclusion, our findings support the view that the suppressor T population derived from cocultures with BPZE1-DC represents conventional T cells that were shifted to a suppressor stage by the activation of 2 distinct, and likely complementary, ADO-generating ectoenzyme networks. Interestingly, and not, to our knowledge, reported previously, these T suppressor cells are equipped with a surface armamentarium that allows metabolization of an extracellular accumulation of both ATP and NAD^+. This strategy may be read as a form of prevention against the detrimental effects of a prolonged inflammatory response. Further experiments may be required with purified T cell subsets to provide insights to the relative contribution of the distinct ectoenzymatic pathways.

Overall, our data argue in favor of BPZE1 as a vaccine candidate to limit the resurgence of pertussis. The notion that a vaccine preparation capable of inducing protective immunity is also capable of generating immune regulation is intriguing and may be considered from 2 different viewpoints. Even though, on one side, BPZE1-induced immune suppression may hamper vaccine efficacy, on the other side, suppression of ongoing T cell responses could be crucial in preventing a response that may be detrimental. A major failure of acellular pertussis vaccines is prevalently linked to the induction of a Th1/Th2 immune response different from that induced by the natural infection or by whole cell vaccines [50]. In contrast, BPZE1 induces both a protective Th1/Th17 response and the activation of a regulatory mechanism to prevent exacerbated inflammation. This may explain why BPZE1 is a potent anti-inflammatory agent, as seen in a number of infectious and noninfectious murine models [33–35], without suppressing adaptive immune responses upon vaccination or infection.

AUTHORSHIP

G.F. designed the study, performed experiments, analyzed data, and wrote the manuscript; I.Sa., K.D., and I.Sc. performed experiments; C.L. provided reagents and contributed to write the manuscript; A.L.H. performed experiments and analyzed data; F.M. provided reagents, contributed to the design of the study and contributed to writing the manuscript; C.M.A contributed to the design of the study and wrote the manuscript. All the authors provided comments to the manuscript.

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DISCLOSURES

The authors declare no competing financial interests.

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