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β2-adrenergic stimulation of dendritic cells favors IL-10 secretion by CD4⁺ T cells

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ABSTRACT

Adrenergic receptor agonists and antagonists are extensively used as drugs in medicine for a broad spectrum of indications. We examined the consequences of β2-adrenergic stimulation of murine dendritic cells (DCs) on CD4⁺ T cell activation. We demonstrated *in vitro* that treatment of LPS-matured DCs with the β2-agonist salbutamol reduced their ability to trigger OT-II T cell proliferation specific for ovalbumin antigen. Salbutamol also induced a decrease in MHC class II molecule expression by DC through Gi protein activation. Co-culture of CD4⁺ T cells with salbutamol-conditioned mature DC impaired TNFα and IL-6 secretion while preserving IL-10 production by T cells. Using a vaccination protocol in mice, we showed that salbutamol favored IL-10-producing CD4⁺ T cells. None of these effects was observed when working with β2-adrenoreceptor deficient mice. Finally, we suggest that β2-adrenergic stimulation of DC could be an interesting way to shape CD4⁺ T cell responses for the purposes of immunotherapy.

KEYWORDS: dendritic cell; CD4⁺ T lymphocyte; salbutamol; β2-adrenergic receptor; IL-10.

INTRODUCTION

Although the immune system has long been considered autonomous, it is now well established that crosstalk exists between the central nervous system and immune cells. Immunity, as part of homeostasis, is indeed tightly controlled by the sympatho-adreno-medullary axis through the release of catecholamines (1). These mediators bind adrenoreceptors (AR) expressed by immune cells. Among AR, dendritic cells (DC) mainly express β2-AR and are thus able to respond to β2-agonists such as salbutamol and fenoterol (2, 3).

DCs are professional antigen-presenting cells controlling both steady-state T cell tolerance and immunity. Thus, modulating DC function appears to be a major challenge for immunotherapy. In particular, promoting a tolerogenic phenotype of DCs could help block autoimmune processes (4, 5). For instance, Giannoukakis *et al.* already produced tolerogenic DCs using antisense oligonucleotides against co-stimulatory molecules and successfully transferred them to diabetic patients (6). Since LPS-matured DCs exposed to salbutamol exhibit decreased secretion of pro-inflammatory cytokines and increased IL-10 secretion (7), the use of salbutamol was proposed for controlling Th1-mediated diseases. Notably, salbutamol was shown to attenuate experimentally-induced inflammatory and autoimmune diseases such as autoimmune arthritis and myocarditis in rodents (8).

DCs shape CD8⁺ and CD4⁺ T cell responses depending on their maturation status, the activation of specialized transcription factors and cytokine production (9). Treatment of LPS-matured DCs using salbutamol was previously shown to decrease protein degradation and peptide presentation on MHC class I molecules (10). This β2-agonist-mediated inhibition of the cross-presentation pathway led to a decrease in subsequent CD8⁺ T cell activation. At the subcellular level, these effects were attributed to a lowering in LPS-induced translocation of the NF-κB complex to the cell nucleus mediated through Gi activation.

Since CD4⁺ T cells are master regulators of immune responses, β2-agonist effects on the DC-CD4⁺ T cell crosstalk were also addressed. Salbutamol's effect on DC-induced CD4⁺ T cell proliferation had been poorly investigated in antigen-specific models; using a Th1 rat cell line and peptide-loaded antigen-presenting cells, Nishii *et al.* demonstrated a decrease in lymphocyte proliferation, whereas, in 2014, Nijhuis *et al.* found no effect of salbutamol on CD4⁺ T cell proliferation using the well-described OT-II model (11, 12). β2-agonist treatment of DCs is also thought to shape CD4⁺ T cell responses. However, although there is a consensus on Th1 inhibition (11, 13), conflicting data were reported concerning either Th2 and/or Th17 differentiation or Treg induction (12–16).

The aim of the current study was to unravel salbutamol's effects on CD4⁺ T cell activation by DCs in an antigen-specific model. Our objective was to extent previous data by testing the implication of the β2-AR, through Gi signaling, not only by using specific inhibitors but also by working with β2-AR KO mice and cells. For this purpose, we worked on GM-CSF differentiated murine hematopoietic precursors commonly called bmDCs (for bone marrow-derived dendritic cells) despite recent controversies (17–20). We analyzed salbutamol's effects on MHC class II molecule expression by LPS-treated DCs and depicted the consequences for their ability to activate antigen-specific CD4⁺ T cells *in vitro*. Lastly, we explored the role of salbutamol in CD4⁺ T cell polarization *in vivo* using a vaccination model in naive C57BL/6J mice. Altogether, our study provides new insights into the cellular and molecular mechanisms by which β2-agonists shape DC-CD4⁺ T cell crosstalk.

MATERIALS AND METHODS*Mice*

H-2b C57BL/6J mice were purchased at Charles River Laboratories (L'Arbresle, France). Rag1^{-/-} OT-II mice originally came from Taconic (Germantown NY, USA). β2-

adrenoreceptor KO mice, initially constructed by B. Kobilka and coll. (21), were provided by M. Barrot (Strasbourg University, France) and backcrossed for 12 generations on the C57BL/6J background. All the β 2-AR KO mice we used were systematically genotyped. All mice were housed at the Oniris Rodent platform and animal experiments were approved by the Pays de Loire Ethical Committee CEEA-PdL (decisions CEEA.2012.92 and CEEA.2012.236).

Chemicals

E. Coli 0111:B4 LPS was purchased from Invivogen and used *in vitro* at a concentration of 1 μ g/ml. Salbutamol hemisulfate and pertussis toxin (PTX) were from Sigma-Aldrich (S^t Quentin-Fallavier, France). ICI 118,551 came from Bio-Techne (Lille, France). Salbutamol, ICI 118,551 and PTX were respectively used at concentration of 1 μ M, 10 μ M and 2 μ g/ml.

Cells

Splenic OT-II CD4⁺ T cells were purified by negative selection using magnetic beads (CD4 T cell isolation kit II, Miltenyi Biotec, Paris, France) and stained using 5,6 carboxyfluorescein succinimidyl ester (CFSE, Fisher Scientific, Illkirch, France) for cell generation tracking.

bmDCs were generated after a 6-day culture in medium supplemented with GM-CSF (J558 supernatant) (22) according to a protocol adapted from Lutz *et al.* (23) and then purified by CD11c⁺ magnetic cell sorting (Miltenyi Biotec). Purity was assessed by flow cytometry and was always > 90%.

MHC class II expression

CD11c⁺ bmDCs were treated with LPS +/- salbutamol +/- ICI 118,551 for eighteen hours and analyzed for I-Ab expression (clone AF6-120.1, BD Biosciences, Le Pont-de-Claix, France) by flow cytometry. Where indicated, cells were pre-treated with PTX for one hour.

In vitro OT-II CD4⁺ T cell activation protocol

CD11c⁺ bmDCs were treated with LPS +/- salbutamol +/- ICI 118,551 while incubated for three hours either with 40 μ g/ml ovalbumin (Endotoxin free OVA, Hyglos, Bernried, Germany) or with 500 nM of the I-Ab-restricted OVA₃₂₃₋₃₃₉ peptide (PolyPeptide Laboratories, Strasbourg, France). Where indicated, cells were pre-treated with PTX or vehicle for one hour. After incubation, DCs were thoroughly washed and co-cultured with CFSE-stained OT-II CD4⁺ T cells in a 96-well plate at a 1:4 ratio in replicate wells. After three days, supernatants were collected to determine cytokine levels by ELISA (BD Biosciences), while T cell proliferation was assessed by flow cytometry after CD4 staining with a PE-Cy7 conjugated anti-CD4 antibody (BD Biosciences).

Flow cytometry

Acquisitions were performed on a FACS Aria flow cytometer (BD Biosciences). For proliferation experiments, we used unstimulated OT-II CD4⁺ T cells to identify the parental population corresponding to undivided cells. CFSE profiles were analyzed using the ModFit LT software (version 4.1, Verity Software House, Topsham, ME) which allows calculation of both the precursor frequency (PF: fraction of parental cells that divided more than twice during the course of the experiment) and the proliferation index (PI:

measurement of the increase in cell number in the culture in the course of the experiment) in each sample. For I-Ab expression, data were computed with the FlowJo software (version 10.1, Ashland, OR).

Vaccination protocol

The protocol used was adapted from den Haan *et al.* (24). Briefly, on day 0, C57BL/6J mice were immunized by intravenous injection of 500 μ g of OVA protein (Sigma-Aldrich) together with 30 μ g of LPS. PBS, for control mice, or salbutamol (200 μ g/mouse) was injected intra-peritoneally on day 0 and day 1. On day 7, splenocytes were restimulated *in vitro* with 40 μ g/ml ovalbumin protein (endotoxin-free OVA, Hyglos), 1 μ g/ml MHC class-I restricted OVA₂₅₇₋₂₆₄ or 100 μ g/ml MHC class-II restricted OVA₂₆₂₋₂₇₆ peptides (Proimmune, Oxford, United Kingdom). After 4 days of culture, supernatants were collected and IL-10 concentration was determined using ELISA (BioLegend, London, United Kingdom; Bio-Techne).

Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 6.0 (La Jolla, CA). For multi-experimental group analysis, data were subjected to the Kruskal-Wallis test followed by Dunn's post test. The Mann-Whitney test was used when only two groups were compared. Only statistically significant differences are indicated on the charts using stars (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$). All experiments were performed at least twice.

RESULTS

β 2-adrenergic stimulation of dendritic cells decreases CD4⁺ T cell proliferation

We analyzed the effects of the β 2-adrenergic agonist salbutamol on DCs' ability to activate CD4⁺ T cell proliferation. *In vitro*-generated, bmDCs were incubated either with OVA protein or with the I-Ab restricted OVA₃₂₃₋₃₃₉ peptide in the presence of LPS with or without salbutamol and/or β 2-AR inhibitor ICI 118,551. After three hours, bmDCs were thoroughly washed and co-cultured with CFSE-stained OT-II CD4⁺ T cells. T cell proliferation was assessed after 3 days (Fig. 1). Representative profiles are shown in Fig. 1A. As expected, LPS-treated DCs induced stronger proliferation of CD4⁺ T cells as compared to immature DCs when incubated with OVA protein or OVA₃₂₃₋₃₃₉ peptide (Fig. 1B). Interestingly, treating mature DCs with salbutamol induced a significant decrease in the proliferation of CD4⁺ T cells. When DCs were directly loaded with the I-Ab restricted OVA peptide, the magnitude of inhibition of CD4⁺ T cell proliferation was stronger than when using OVA protein. The inhibitory effect of salbutamol on T cell proliferation was reverted when ICI 118,551 was added at the time of bmDC treatment. Of note, in the absence of salbutamol, we did not detect any effect of ICI 118,551 (data not shown). When using DCs isolated from β 2-AR deficient mice (Fig. 1C), salbutamol treatment did not impair T cell proliferation, confirming a β 2-adrenoreceptor-dependent phenomenon.

Figure 1: $\beta 2$ -AR stimulation of bmDCs decreases CD4⁺ T cell proliferation.

A, Representative CFSE profiles of OT-II CD4⁺ T cells co-cultured with DCs for three days. C57BL/6J bmDCs were pre-incubated for three hours with the I-Ab restricted OVA₃₂₃₋₃₃₉ peptide and either left untreated or matured with LPS +/- salbutamol +/- ICI 118,551. Arrowheads identified the peaks automatically found by the peak finder tool of Modfit LT software. PF, precursor frequency; PI, proliferation index. B, CFSE-stained OT-II CD4⁺ T cells were co-cultured for 3 days with wild-type bmDCs previously incubated with soluble OVA protein or with the cognate I-Ab restricted OVA₃₂₃₋₃₃₉ peptide +/- LPS, salbutamol and ICI 118,551. Precursor frequencies are shown. One experiment representative of four (for protein) and three (for peptide) is shown. C, Similar experiments were performed using peptide-loaded $\beta 2$ -AR^{-/-} bmDCs. One experiment representative of three is shown.

$\beta 2$ -adrenergic stimulation of dendritic cells lowers I-Ab expression in a Gi manner

We hypothesized that salbutamol treatment disturbed the early DC signals needed for T lymphocyte activation. Since salbutamol treatment was previously shown to only induced a slight decrease in CD40, CD80 and CD86 expression ((10) and not shown), we explore herein salbutamol's effects on MHC class II molecule expression by LPS-matured DCs. Surface I-Ab expression on bmDCs was assessed using flow cytometry. As expected, LPS treatment enhanced the I-Ab^{high} cell proportion in both $\beta 2$ -AR^{+/+} (Fig. 2A) and $\beta 2$ -AR^{-/-} DCs (Fig. 2C). Salbutamol treatment markedly impaired LPS-induced I-Ab^{high} cell proportion increase in bmDCs while lowering I-Ab^{high} expression at the cell surface of I-Ab^{hi} cells (Fig. 2 A and B). This effect was $\beta 2$ -adrenoreceptor-mediated as it was abolished when $\beta 2$ -AR^{+/+} cells were treated with ICI 118,551 or when working with $\beta 2$ -AR^{-/-} DCs (Fig. 2C). Although the $\beta 2$ -adrenoreceptor has historically been demonstrated to signal mainly through Gs protein, it was also shown to activate Gi protein (10). In this context, we tested the effects on I-Ab expression of pre-incubating DCs with the Gi disruptor pertussis toxin (PTX). As shown in Fig. 2D, in the presence of PTX, the inhibitory effect of salbutamol on I-Ab expression was not observed anymore.

Figure 2: Salbutamol reduces I-Ab expression by DCs in a Gi dependent manner.

$\beta 2$ -AR^{+/+} (A, B and D) or $\beta 2$ -AR^{-/-} (C) bmDCs were treated as indicated, stained for I-Ab and analysed by flow cytometry. I-Ab^{high} cell proportion and rMFI (MFI related to the one of the isotype) are shown. One experiment representative of three is shown, except for PTX (one upon two).

Salbutamol treatment of dendritic cells inhibits pro-inflammatory cytokine secretion while preserving IL-10 secretion by CD4⁺ T cells *in vitro*.

We next investigated the consequences of salbutamol treatment of DC on CD4⁺ T cell polarization. To this aim, we measured cytokine secretion in supernatants obtained after a three-day co-culture of OT-II CD4⁺ T cells with differentially treated DCs (Fig. 3). Of note, we did not detect IL-4 and IL-17 cytokines in any of the test conditions. As expected, when exposed to LPS-matured DCs, CD4⁺ T cells secreted higher levels of TNF α , IL-6, IFN γ and IL-10. β 2-adrenergic stimulation of LPS-matured DCs significantly decreased TNF α and IL-6 secretion by CD4⁺ T cells and this effect was partly reverted by the addition of ICI 118,551. Albeit not statistically significant, salbutamol treatment of DCs also tended to lower IFN γ secretion in co-cultures. In contrast, IL-10 secretion by CD4⁺ T cells slightly increased when mature DCs were treated with salbutamol. This effect on IL-10 secretion was β 2-AR-mediated as it was abolished by ICI 118,551. These effects were not observed when T cells were co-cultured with β 2-AR^{-/-} DCs (Suppl. Fig. 1). To sum up, salbutamol treatment of DC prevented the LPS-induced Th1 polarization of CD4⁺ T cells and seem to maintain IL-10 secretion.

Figure 3: Salbutamol treatment of DCs inhibits pro-inflammatory cytokine production and maintains IL-10 secretion by CD4⁺ T cells. OT-II CD4⁺ T cells were co-cultured with bmDCs pre-incubated with OVA₃₂₃₋₃₃₉ peptide +/- LPS, salbutamol, ICI 118,551. At day 3, supernatants were collected and tested for cytokine secretion. One experiment representative of three is shown, except for TNF- α and IL-6 (one upon two).

Salbutamol enhances vaccination-induced IL-10 secretion by CD4⁺ T cells

We then addressed the question of the effect of salbutamol on IL-10 secretion by CD4⁺ T cells *ex vivo* using a vaccination protocol previously demonstrated to elicit Ag-specific, IL-10-producing CD4⁺ Tr1 cells (24). β 2-AR^{+/+} and β 2-AR^{-/-} mice were immunized with LPS and OVA protein then treated with salbutamol or vehicle. IL-10 secretion was finally measured after re-stimulating splenocytes *in vitro*. We demonstrated

that salbutamol treatment significantly increased IL-10 production by OVA protein re-stimulated splenocytes (Fig. 4A), with IL-10 levels ranging from 323 to 1030 pg/ml for vehicle-treated and from 399 to 1532 pg/ml for salbutamol-treated, β 2-AR^{+/+} mice. In contrast, salbutamol administration did not change IL-6 and IFN γ secretions (Suppl. Fig. 2); of note, TNF α was not detected in these conditions. Interestingly, the specific increase in IL-10 production was not observed in β 2-AR^{-/-} mice.

Next, to check whether the salbutamol-induced increase in IL-10 secretion relied on CD4⁺ T cells or not, we measured IL-10 production following stimulation by class I- or class II-restricted OVA peptides. As shown on Fig. 4B, the increase in IL-10 secretion induced by salbutamol was observed not only when splenocytes were restimulated with OVA protein, but also with the MHC class II-restricted OVA₂₆₂₋₂₇₆ peptide. Importantly, no IL-10 secretion enhancement was observed when cells were restimulated with class-I restricted OVA₂₅₇₋₂₆₄ peptide. These observations ruled out the involvement of cells other than CD4⁺ T cells in the phenomenon observed. Thus, we presume that salbutamol promoted IL-10-producing CD4⁺ T cells *in vivo*.

DISCUSSION

Dendritic cells drive CD4⁺ T cell polarization, a key-step process leading either to immunity or to tolerance, mainly related to the DC maturation stage and cytokine production (9). In this respect, DCs integrate multiple signals from the tissue microenvironment, including hormones and neurotransmitters. At steady state, the maintenance of immune tolerance may result from an active process involving homeostatic mediators such as catecholamines. Catecholamines may also participate in restoring immune homeostasis in case of immune challenge. Catecholamines include hormones and neurotransmitters that can bind to numerous receptors. Among them, the β 2-adrenoreceptor, well-represented on DCs, can be activated by endogenous compounds (epinephrine and norepinephrine) as well as drugs such as salbutamol.

We report herein on the modulating effect of the β 2-agonist salbutamol on CD4⁺ T cell activation induced by LPS-matured DCs. Since the strength of CD4⁺ T cell stimulation largely relies on the first signal provided by MHC-II/peptide complexes/TCR interaction, we explored salbutamol's effects on surface MHC class II molecule expression by LPS-matured DCs *in vitro*. We demonstrated a salbutamol-induced decrease in surface I-A_b expression, not observed in β 2-AR^{-/-} DCs, hence indicating a β 2-AR-dependent mechanism. Accordingly, recently, β 2-adrenergic stimulation of rat bmDCs with isoprenaline was already shown to reduce MHC class II molecule expression (25). In our experiment, pertussis toxin addition prevented salbutamol effects on MHC class II molecule expression, thereby confirming a Gi-mediated effect. While signalling through β 2-AR classically involves Gs protein, the coupling of the receptor to Gi protein has already been demonstrated in different cell types including DCs (10, 26, 27). While the molecular basis of selective coupling of β 2-adrenoreceptor to Gs and/or Gi proteins remains largely unknown, β 2-AR coupling to Gi protein is generally accepted to result from a PKA-dependent switch from Gs protein (28, 29).

Figure 4: Salbutamol favors IL-10-producing CD4⁺ T cells after vaccination. β2-AR^{+/+} (filled symbols) and β2-AR^{-/-} (open symbols) mice were vaccinated with OVA protein (A, B) or peptides (B). Four days later, spleen cells were harvested 7 days later and restimulated *in vitro* with OVA protein (A, B) or peptides (B). Four days later, supernatants were collected and assayed for IL-10 concentrations. In each graph, results are expressed relatively to the mean of the “OVA protein-restimulated, vehicle-treated” group. Each symbol represents one mouse and pooled data from two independent experiments are shown.

In DCs, the β2-agonist effects were rather shown to result from a PKA-independent mechanism (3, 10). Whether salbutamol induces β2-AR/Gi coupling directly or after Gs eviction following kinase-dependent β2-AR phosphorylation remains to be addressed. Of note, in cardiomyocytes, the coupling of β2-AR to Gi was shown to result from GRK2-mediated phosphorylation of the receptor (30). At present, the molecular mechanisms resulting from Gi activation by β2-agonists in LPS-treated DCs are not fully understood, but β2 adrenoreceptor stimulation might activate MAP kinases and PI3K signalling cascades (29). Still, NF-κB and AP-1 transcription factors were previously identified as downstream targets of β2-AR signaling in DC (3, 10).

Expression of MHC class II molecules on antigen-presenting cells is tightly regulated at the transcriptional and post-transcriptional levels. The MHC class II transactivator (CIITA) is the master regulator of MHC class II gene expression (31). In bmDCs, LPS-induced MHC class II molecule increase was shown to rely upon the upregulation of *I-Ab* gene transcription through an AP-1 enhancer (32). NF-κB was also previously proposed as being involved in LPS-induced MHC class II expression in myeloid cells, since removal of the NF-κB box in the promoter reduced LPS-induced MHC class II expression (33). Thus, we suggest that salbutamol may impair LPS-induced MHC class II expression by dampening NF-κB and/or AP-1 activity.

We also demonstrated that salbutamol impaired the ability of antigen-loaded LPS-treated bmDCs to activate CD4⁺ T cell proliferation. Although previously explored in the same model by Nijhuis *et al.* (12), this inhibitory effect of salbutamol on DC-induced-CD4⁺ T cell proliferation was not reported. Indeed, using a higher dose of OVA protein (250 μg/ml), OT-II CD4⁺ T cell proliferation was found unchanged by salbutamol treatment of DCs. Since T cells proliferated vigorously in their experiments, the salbutamol-induced inhibition of proliferation monitored at day 4 might have been overlooked. Interestingly, an agonist of α2-adrenoceptors, known to be coupled with Gi upon activation, was shown to induce the same effects as those observed using salbutamol, *i.e.* a reduction in I-Ab expression by DCs combined with a decreased ability to induce CD4⁺ T cell proliferation (34).

There is strong evidence that β2-agonists prevent Th1 development in humans and mice (7, 13), while diverse data were reported for other CD4⁺ T cell polarization subtypes. In particular, Kim and Jones previously reported an epinephrine-mediated induction of Th2 and Th17 cells using polyclonal

stimulation of T cells (16). Under their conditions, Th17 induction relied on β2-adrenoreceptor stimulation while Th2 polarization did not. In contrast, we detected neither IL-4 nor IL-17 secretion by OT-II cells in our experiments. Nevertheless, salbutamol treatment of LPS-matured DCs induced a decrease in pro-inflammatory cytokine secretions (TNFα and IL-6) while slightly increasing IL-10 secretion, which is consistent with recently published data by Nijhuis *et al.* (12). In the aforementioned study, salbutamol was suggested to imprint bmDCs to stimulate Foxp3⁺ regulatory T cell differentiation. It is noteworthy that, in these experiments, the salbutamol-induced effect on Foxp3⁺ T cell generation was significant only when TGFβ was exogenously added during the co-culture. In agreement with Nijhuis *et al.*, we also found that TGFβ production by bmDCs was not influenced by β2-AR activation (data not shown). Thus, in our *in vitro* settings, it seems unlikely that naive T cells underwent Foxp3⁺ Treg cell differentiation due to TGFβ signaling.

In vaccination experiments, we demonstrated an increased secretion of IL-10 by *ex vivo* re-stimulated CD4⁺ T cells from salbutamol-treated LPS/OVA vaccinated mice. In these *in vivo* experiments, we cannot exclude that the observed effects result from both direct and DC-mediated effects of salbutamol on CD4⁺ T cells. Indeed, CD4⁺ T cells also express β2-AR and respond to β2-agonists (35). Nevertheless, we evidenced an increase in IL-10 secreting CD4⁺ T cells, while finding similar percentages of Foxp3⁺ Treg cells in vehicle and salbutamol-treated mice (data not shown). In similar experimental conditions, these IL-10-secreting CD4⁺ T cells were identified as suppressive Tr1 cells (24). Among regulatory T cells, Tr1 cells are defined by the co-expression of c-Maf and AhR transcription factors as well as secretion of IL-10 and IL-21 (36, 37). Although further experiments are required to characterize these IL-10-secreting T cells, our data suggest that salbutamol treatment promotes Tr1 generation rather than Foxp3⁺ Treg generation.

Future studies are warranted to evaluate the potential benefit of using β2-agonists to shape CD4⁺ T cell responses towards a Tr1 phenotype. Tr1 cells give rise to a growing interest in treating autoimmune diseases such as multiple sclerosis or type 1 diabetes. Pharmacological approaches to expand autoantigen-specific Tr1-like cells are still lacking. In this context, using Gi-biased β2-agonists could help blunt the autoimmune process in human patients.

ETHICAL APPROVAL

All applicable international and national guidelines for the care and use of animals were followed. All procedures performed in studies involving mice were in accordance with the ethical standards of the institution at which the studies were conducted.

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SUPPLEMENTAL FIGURES

Supplemental Figure 1: Salbutamol effect on cytokine production by CD4⁺ T cells is not observed when cocultured with $\beta 2\text{-AR}^{-/-}$ DCs.

OT-II CD4⁺ T cells were co-cultured with $\beta 2\text{-AR}^{-/-}$ bmDCs pre-incubated with OVA₃₂₃₋₃₃₉ peptide +/- LPS and salbutamol. At day 3, supernatants were collected and tested for cytokine secretion.

Supplemental Figure 2: Salbutamol does not affect IL-6 and IFN γ production after vaccination.

$\beta 2\text{-AR}^{+/+}$ mice were vaccinated with OVA protein in the presence of LPS and treated with vehicle or salbutamol. Spleen cells were harvested 7 days later and restimulated *in vitro* with OVA protein. Four days after, supernatants were collected and assayed for IL-6 and IFN γ concentrations. In each graph, results are expressed relatively to the mean of the vehicle-treated group. Each symbol represents one mouse and pooled data from two independent experiments are shown.