Original Research Paper

# Effects of Unilateral Cervical Vagotomy on Murine Dendritic Cells

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**Abstract:** The cholinergic anti-inflammatory pathway has been demonstrated to be an important mechanism that modulates several inflammatory diseases. This modulation occurs upon activation of the vagus nerve, which modulates splenic macrophages and lymphocytes through the predominant neurotransmitter, Acetylcholine (ACh). We hypothesized that this modulation could also take place in dendritic cells, as these cells represent a link between the innate and adaptive immune responses. Bone Marrow-Derived Dendritic Cells (BMDCs) were generated from mice subjected to a unilateral cervical vagotomy and the phenotypes and functions of the BMDCs were evaluated. No significant effects were found in BMDCs from vagotomized mice compared to cells from the control group. The specific immune response was evaluated with the Delayed-Type Hypersensitivity (DTH) skin test using Keyhole Limpet Hemocyanin (KLH) as an antigen and the peak of the response in the vagotomized animals was delayed compared to the control group. It is possible that the Dendritic Cells (DCs) can be modulated by the vagus nerve in other specific sites or under challenging conditions, but our work suggests that immune cells could be modulated by the components of another cholinergic anti-inflammatory pathway or by another neuro-immune pathway interaction.

**Keywords:** Dendritic Cells, Vagus Nerve, Cholinergic Anti-Inflammatory Pathway, Neuroimmunology

## Introduction

The cholinergic anti-inflammatory pathway is an important neuro-immune communication mechanism in the maintenance of homeostasis. Specifically, the electrical or pharmacological stimulation of the vagus nerve modulates pro-inflammatory responses by binding its main neurotransmitter, Acetylcholine (ACh), into the nicotinic alpha-7 nicotinic receptors (α7nAChR) present on immune cells (Pavlov and Tracey, 2005). Splenic macrophages are among these immune cells and are targets of the cholinergic anti-inflammatory pathway in models of chronic intestinal disease (Ghia *et al*., 2006) and in endotoxemia, where the production and secretion of Tumor Necrosis Factor

(TNF) by these cells is inhibited (Borovikova *et al*., 2000). In lymphocytes, it has been shown that subdiaphragmatic vagotomy increases the production of pro-inflammatory cytokines and proliferation of these cells *ex vivo* (Karimi *et al*., 2010). Other immune cells such as Dendritic Cells (DCs) also possess cholinergic receptors (Kawashima *et al*., 2007), are involved in inflammatory processes and can activate other cells by making contact with receptors and secreting pro-inflammatory factors. Several *in vitro* studies have demonstrated the controversial effects of cholinergic agonists on these cells, sometimes showing anti-inflammatory (Nouri-Shirazi and Guinet, 2003) or pro-inflammatory effects (Aicher *et al*., 2003; Gao *et al*., 2007; 2011). However, little is known about the

effects of the vagus nerve on myeloid precursor cells or the subsequent phenotypic and functional outcomes of the Bone Marrow-Derived Dendritic Cells (BMDCs). In the present study, we aimed to determine the constitutive influence of the vagus nerve on the attributes of generated BMDCs using a partial vagotomy technique.

## Materials and Methods

### Animals

Eight to twelve week old female mice from the C57BL/6 and OT-II (animals with transgenic T cell receptors specific for ovalbumin-OVA) strains were used. The animals were housed in rooms with temperature (22±2ºC), humidity (65-70%) and artificial light control (12 h light/12 h dark cycle, lights on at 7:00 am) in polypropylene cages with *ad libitum* access to Nuvilab® rodent chow (Nuvital, Colombo, PR) and filtered water. Sterile wood shavings were used as bedding for the animals. The mice were obtained from the Department of Pathology, School of Veterinary Medicine and Animal Science, University of Sao Paulo, where they were also maintained for all the experiments. The animals were used following the ethical standards and procedures related to use of laboratory animals from the FMVZ Ethics Committee-USP (2330/2011).

### Surgical Procedures

The unilateral cervical vagotomy technique was based on and adapted from van Westerloo *et al*. (2005). Briefly, the animals were subjected to analgesia with a subcutaneous injection of 0.1 mg kg$^{-1}$ of fentanyl and anesthetized intraperitoneally with ketamine and xylazine at doses of 0.05 mg g$^{-1}$ and 0.02 mg g$^{-1}$ body weight, respectively. A ventral cervical midline incision was made to expose the right vagal trunk, which was separated from the carotid artery and transected. The skin synthesis was performed with simple interrupted sutures using a 4-0 nylon thread. In the *sham* animals, the surgical intervention was similar to that used in the denervated group, but the nerve was not transected. Seven days after surgery, the animals were euthanized for the *ex vivo* experiments, or used in the *in vivo* experiment.

### Generation of Bone Marrow-Derived Dendritic Cells

The method used to generate BMDCs was adapted from Lutz *et al*. (1999). Briefly, the bone marrow was taken from the femurs and tibiae of mice by washing the inside of the bones with RPMI-1640 medium (Gibco, Grand Island, NY) using sterile needles. The red blood cells were lysed with ACK lysis buffer (NH$_4$Cl 0.15M, KHCO$_3$ 10 mM, EDTA 0.1 mM). The cells were adjusted in a hemocytometer and maintained in dendritic cell culture medium (DC-RPMI), which consisted of RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS-LGC Biotechnology Cotia, SP), penicillin (100 U mL$^{-1}$) (Gibco, Grand Island, NY), streptomycin (100 mg mL$^{-1}$) (Gibco, Grand Island, NY), L-glutamine (Gibco, Grand Island, NY) (2 mM), 2-mercaptoethanol (Gibco, Grand Island, NY) (50 μM) and 3% supernatant of the NIH/3T3 fibroblast lineage containing the murine recombinant GM-CSF. The cells were seeded in a 24-well plate at a concentration of 7.5×10$^5$ cells/well and kept in a 5% CO$_2$ incubator at 37°C. On the third day of culture, 1 mL of RPMI-DC was added to all wells. On the sixth day, half of the culture medium was removed from the wells, centrifuged, then the cells were resuspended in RPMI-DC and returned to the plate. On the seventh day, to promote the maturation of the cells, 1 μg mL$^{-1}$ Lipopolysaccharide (LPS) was added during the last 24 h of culture.

### Assessment of Co-stimulatory Molecules by Flow Cytometry

To measure the expression of co-stimulatory molecules, 1×10$^6$ BMDCs were incubated for 30 min at 4°C in the dark with fluorescein isothiocyanate (FITC)-conjugated anti-CD11c (clone N418) (Biolegend), phycoerythrin (PE)-conjugated anti-MHCII (clone AF6-120.1) (BD Biosciences), Allophycocyanin (APC)-conjugated anti-CD80 (clone 16-10A1) (BD Biosciences) and Peridinin Chlorophyll Protein (PerCP)-conjugated anti-CD86 (clone GL-1) (Biolegend). All antibodies were diluted 1:100 in PBS. The cells were washed once and the fluorescence was acquired using a FACSCalibur flow cytometer (Becton Dickinson).

### Endocytosis Assay by Flow Cytometry

To assess the endocytic capacity of the BMDCs, 2×10$^5$ cells were incubated with the monoclonal antibody anti-CD11c (BioLegend, San Diego, CA) and killed *Staphylococcus aureus* were stained with Propidium Iodide (SAPI) for 1 h at 37°C. The endocytosis was stopped by the addition of 3 mM cold Ethylenediaminetetraacetic Acid (EDTA). The fluorescence was then acquired on a FACSCalibur flow cytometer (Becton Dickinson).

### In Vitro Stimulation of OVA-Specific T Cells

After the generation of BMDCs, 1×10$^6$ cells mL$^{-1}$ cells were incubated for 4 h with 1 mg mL$^{-1}$ of grade

II OVA (Sigma-Aldrich, St. Louis, MO) in a 5% $CO_2$ incubator at 37°C for internalization of the protein. Then, 1 µg mL$^{-1}$ of LPS was added for 24 h to induce maturation of the cells. Lymphocytes were collected and separated from the spleens of OT-II animals with Ficoll-Paque™ PLUS (GE Healthcare Life Sciences) according to the manufacturer's recommendations. The cells were then stained with Diacetate Succinimidyl Carboxyfluorescein Ester (CFSE) (5 µg mL$^{-1}$) for 20 min at 37°C. The cells were washed and plated at a concentration of $2 \times 10^5$ cells/well in co-culture with the BMDCs at the following ratios of stained cells to BMDCs: 5:40,000; 25:8000; 50:4000; and 100:2000. The experiment was conducted in 96-well U bottom plates, which were incubated in 5% $CO_2$ at 37°C for 4 days. At the end of this period, the cells were collected and the fluorescence was acquired on a FACSCalibur flow cytometer (Becton and Dickinson). For the fluorescence data analysis, the percentage (%) of cell divisions was considered.

*Statistical Analysis*

All results were expressed as the means ± SEM. The significant differences between groups were calculated using Student's *t*-test. The results were considered significant when $p < 0.05$. The statistical analyses and the resulting graphs were generated using the Tree Star FlowJo software version 7.6.1 for Windows and GraphPad Prism version 6.0 for Windows.

# Results

*Right-sided Cervical Vagotomy does not Alter the Expression of Surface Molecules of BMDCs Neither Phagocytosis of SAPI*

The expression of surface molecules such as MHC-II and co-stimulatory molecules such as CD80 and CD86 are related to antigen presentation by DCs to naïve T cells. Therefore, we decided to investigate whether the right-sided cervical vagotomy (Vgx) was able to modify the expression of these molecules in BMDCs. Fig. 1a shows that the Vgx was not able to alter the expression of MHC-II and co-stimulatory molecules (CD80 and CD86) in the BMDCs treated with LPS in vagotomized animals relative to the *sham* controls. These results showed that the Vgx was not able to interfere with the generation (data not shown) and maturation of DCs. Still, it is important to consider that immature DCs have a greater endocytic capacity than mature cells (Banchereau *et al.*, 2000). Therefore, we evaluated the ability of BMDCs derived from animals with Vgx to perform SAPI endocytosis. As shown in Fig. 1b, the fluorescent intensity of

SAPI$^+$DCs in vagotomized animals did not differ from its *sham* counterparts. Because the results indicated that vagotomy did not have a significant effect on the function of immature BMDCs or their maturation, we decided to investigate whether the Vgx was able to alter the function of mature DCs.

*Right-sided Cervical Vagotomy does not Alter the Ability of LPS-Stimulated DCs to Stimulate OVA-Specific Proliferation of T Cells*

One of the main characteristics of mature DCs is the stimulation of differentiation and proliferation of T cells. Thus, we investigated whether BMDCs of the animals receiving Vgx were able to stimulate a proliferative response in the OVA-specific T cells. To do this, the immature DCs were incubated with OVA, stimulated with LPS and co-cultured with T cells from OT-II mice. As shown in Fig. 2, the DCs were able to stimulate T cell proliferation in the 50 and 100 ratios, but no significant differences were found between the cells of the vagotomized animals compared to the *sham* group. These results showed that the vagotomy was not able to change either the function of BMDCs stimulated with LPS or their capacity to stimulate a T cell response.

*The Right-Sided Cervical Vagotomy does not Alter the DTH Response but Interferes with the Kinetics of the Response*

As one of our goals was to assess the antigen presentation of DCs in vagotomized mice, we used DTH as a model of antigen presentation *in vivo* because the DCs are important cells in the initiation of adaptive, antigen-specific cellular responses (Guinet *et al.*, 2004). Fig. 3 shows that both group of animals presented paw edema starting at 24 h after the stimulus. Though the differences were not statistically significant, the profiles of the responses seemed to differ between the groups. Compared to the *sham* group, the Vgx group showed a smaller increase in swelling during the first 24 h, which peaked at 48 h when the swelling of the sham animals was already decreasing. This result suggests that the kinetics of the inflammatory response were altered.

# Discussion

The results obtained in our study demonstrated that the partial absence of the vagus nerve did not promote profound alterations in the phenotype or endocytosis of *in vitro* cultivated LPS-stimulated BMDCs. In contrast, there are some studies in the literature that

points that nicotine, a cholinergic agonist, could promote diverse effects on BMDCs *in vitro*. One study has demonstrated that nicotine was able to elevate the expression of MHC-II, co-stimulatory molecules such as CD86 and CD40 and the adhesion molecules LFA-1 and CD54 in addition to reducing endocytosis (Aicher *et al.*, 2003). Another study also reported a role for nicotine in stimulating DCs, which showed anti-tumor effects (Gao *et al*, 2007). Conversely, the opposite effects have been demonstrated (Guinet *et al*., 2004), with a reduction of the co-stimulatory markers and an increase of receptor-mediated endocytosis by nicotine-treated

cells. It is possible that the stimulation of the α-7 cholinergic receptors of the BMDCs could promote or inhibit DC maturation, but the reduction of cholinergic agonists used in our model does not necessarily lead to either effect.

Similarly to the results described above, we could not observe any alteration to the BMDC-induced T cell proliferation response. On the other hand, this has been observed by some other groups, who have reported either an increase (Aicher *et al*., 2003; Gao *et al*., 2007; 2011) or a decrease (Nouri-Shirazi and Guinet, 2003; Guinet *et al*., 2004) in lymphocyte proliferation induced by nicotine-stimulated DCs.
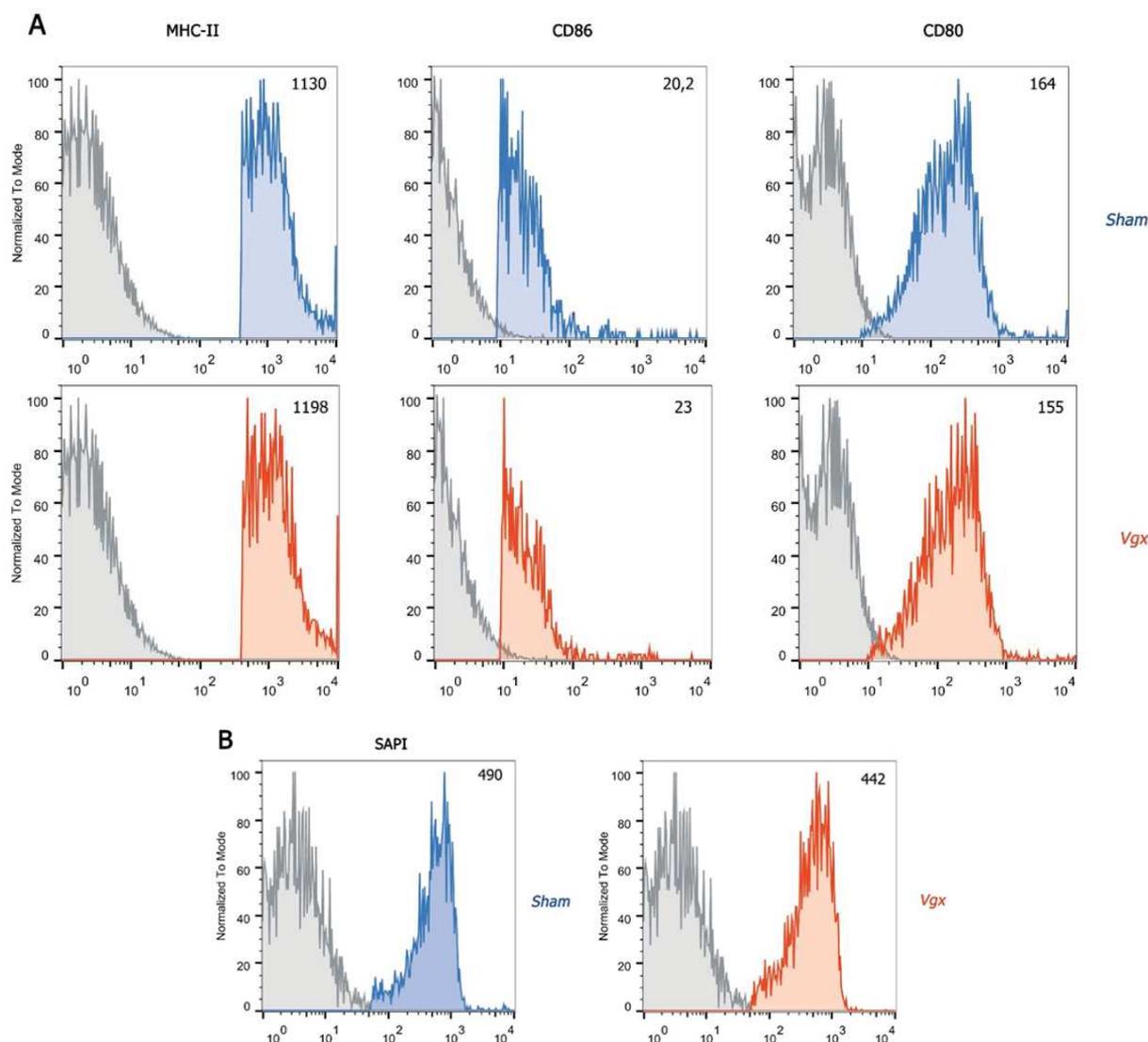


Fig. 1. (a) The Median Fluorescent Intensity (MFI) of MHC-II and the co-stimulatory molecules CD80 and CD86 and (b) the MFI of SAPI by BMDCs from mice subjected either to right-sided unilateral vagotomy (Vgx) or just exploratory surgery (sham). The light grey histogram represents an unstained control. The figures are representative of one of two independent experiments (n = 3 animals/group/experiment) with similar results. The values are shown as the means ± SEM of the samples (p<0.05-Student's *t* test)
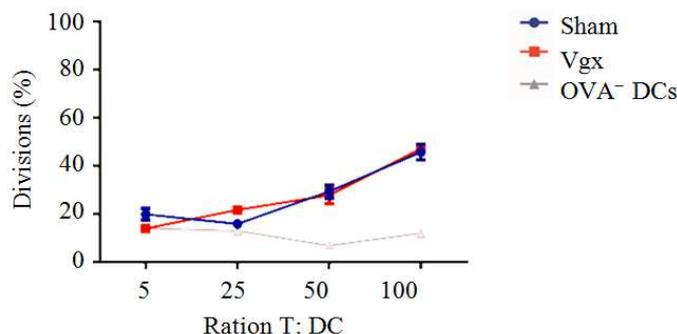
Fig. 2. Curve of the % of divisions by OVA-specific T cells when co-cultured with BMDCs from mice subjected either to right-sided unilateral vagotomy (Vgx) or just exploratory surgery (sham). The figure represents one experiment (n = 10 animals/group). The values are shown as the means ± SEM of the replicates for each mouse (p<0.05 -Student's *t* test)
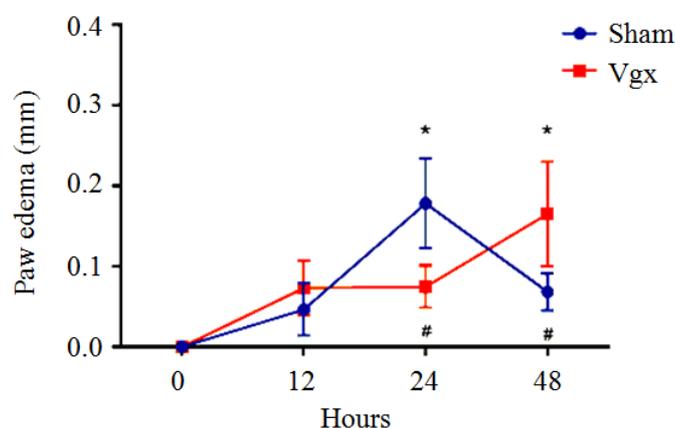


Fig. 3. A DTH curve depicting the magnitude of the swelling in the paws of mice subjected either to right-sided unilateral vagotomy (Vgx) or just exploratory surgery (*sham*) that were either immunized or challenged with KLH. The figure represents one of two independent experiments (n = 3 animals/group/experiment) with similar results. The values are shown as the means ± SEM of the measurements. (*) p<0.05 Vs sham group, hour 0. (#) p<0.05 Vs Vgx group, hour 0 (Student's *t*-test)

The current literature documenting the effect of cholinergic agents on DCs as well as the relationship between these cholinergic agents, the vagus and DCs is controversial. Since the biological effects of nicotine or neurotransmitters is dependent on dose and exposure time, these controversial effects can be attributed to differences in experimental design, species, exposure time and particularly the levels of nicotine and/or neurotransmitters in contact with the DCs. This last point is especially true because DCs are finely controlled by nicotine and neurotransmitters in the local environment, as demonstrated in the work of Hu *et al.* (2012), in which different doses of nicotine followed by LPS provided different outcomes in DCs.

Most studies that have investigated the effects of cholinergic agents on DCs were performed with BMDCs treated with agonists *in vitro*. By contrast, our study was conducted with BMDCs that were influenced by the partial lack of innervation *in vivo,* making direct comparisons difficult *per se*. Taking the results of our *in vitro* studies together, we could speculate that the effects of the cholinergic pathway on DCs may occur on the tissues containing DCs and not on the precursors of these cells. In a study using a model of colitis, a subdiaphragmatic vagotomy was able to increase the expression of MHC-II and pro-inflammatory cytokines in CD11c+ cells in the spleen even though the vagotomy was not able to alter the expression of the co-stimulatory molecules CD80 and CD86 (Ji *et al.*, 2014). This is a similar result to that obtained in the present study. Munyaka *et al.* (2014), using the same model of colitis, also showed an increase of pro-inflammatory cytokines produced by T cells co-cultured with spleen DCs from animals submitted to subdiaphragmatic vagotomy. In pathological situations, such as colitis, the vagus nerve may exert an anti-inflammatory tonic modulation on the dendritic cells of the spleen. However, it is possible that the effects of this tonic modulation are not strong enough

to lead to significant changes in the BMDCs precursor cells, as the DCs generated from these cells showed similar phenotypes and functions to those from animals with intact vagus nerve.

In this study, we used the DTH test to further explore the possibility that the partial absence of the vagus nerve may interfere with the immunization process and the subsequent recall of this immunization. The DTH response is an inflammatory reaction in which a site challenged with an antigen that was used in a previous immunization is infiltrated by memory T lymphocytes and macrophages, which primarily mediate the inflammatory reaction (Szabo *et al.*, 2003). In the present study, we used KLH with Freund's adjuvant as the antigen. Immunization is a fundamental process for the effective establishment of a DTH. In the immunization process, the antigenic agent is inoculated in the presence of an adjuvant that produces a local inflammatory response mediated by cells of the innate immune system. One of the populations of innate cells that are present at the site of this inflammation is the DCs (Banchereau *et al.*, 2000). Once the DCs come into contact with the inoculated antigen, these cells migrate to the lymphoid organs and initiate antigen-specific adaptive cellular responses (Macia *et al.*, 2006). As one of our goals was to evaluate the antigen presentation of DCs from vagotomized animals, it seemed relevant to use the DTH test as a model of antigen presentation *in vivo*. As a result, we expected that the vagotomized animals would have an exacerbated inflammatory response compared to the sham group, but this effect was not observed. Interestingly, the data tended to show that the peak response in the vagotomized animals was delayed and was possibly maintained for longer relative to the sham group, but these effects need further investigation using histological and cytokine studies because other immune cells may be involved.

In our study, the lack of observable effects in BMDCs cultivated from vagotomized mice and in our *in vivo* experiments could be perhaps explained by another mechanisms involved, such as the recovery time after surgery for animals that may be relevant to the cholinergic anti-inflammatory pathway. As demonstrated by Ghia *et al.* (2007), the effects of subdiaphragmatic vagotomy decrease over time and are eventually offset by other anti-inflammatory mechanisms, such as induction of Treg cells and the secretion of IL-10. Although our model of vagotomy was different, our experiments were performed a week after vagotomy, therefore, it is possible that these compensatory mechanisms were already activated in our mice. Moreover, as in unilateral cervical vagotomy, one of the cervical branches of the vagus nerve is preserved so it is not possible to rule out that this branch over time pass to exercise some of the functions of the branch transected and, therefore, the effects were not observable because the homeostasis was reestablished. We could hypothesize that only the acute activation of

cholinergic receptors leads to more evident changes in DCs and that the tone of the vagal modulation on these cells causes only discrete biological effects, being shown only in the case of vagal activation front a signaling of the immune system.

Still, more recently was been demonstrated that instead of cholinergic, adrenergic agonists were able to increase endocytosis and IL-10 and reduce the production of IL-6, IL-12p70 and IL-23 in addition to polarize the T cell response for Tregs, suggesting that the effects on DCs are mediated by catecholamines and not by acetylcholine (Nijhuis *et al.*, 2014). In fact, it is known that the vast majority of lymphoid organs, such as spleen and bone marrow, receive sympathetic innervations only and that the splenic nerve is an essential component of the cholinergic anti-inflammatory pathway. Martelli *et al.* (2014a) demonstrated that the splanchnic nerve and not the vagus, activate the splenic nerve producing anti-inflammatory responses. On another work, they hypothesized that communication between the vagus and the splenic nerve is not by nervous pathways, but by immune cells that are activated at sites of vagal innervation and migrate to the spleen where they release neurotransmitters that activate the sympathetic splenic fibers (Martelli *et al.*, 2014b).

## Conclusion

In summary, our results do not support the hypothesis of a constitutive modulation of the vagus nerve on the functional characteristics of BMDCs. However, we cannot dismiss that this modulation may occur at other sites, or in certain pathological situations. Moreover, studies that lead to a better understanding of the components involved in the cholinergic anti-inflammatory pathway may provide clues concerning how this system can affect the behavior of DCs, either directly, or indirectly through other immune cells and factors secreted by those.

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## Author Contributions

**Daniel Sanzio Gimenes da Cruz:** Conducted all the surgical procedures and experiments, participated in the

design of the study, statistical analysis, writing and the discussion of the manuscript.

**Ana Paula Nascimento de Lima:** Participated in the experiments, in the design of the study, writing and the discussion of the manuscript.

**Patrícia Benites Gonçalves da Silva:** Participated in the experiments, in the design of the study, writing and the discussion of the manuscript.

**João Palermo-Neto:** Participated in the design of the study, financial support and the discussion of the manuscript.

**Cristina Massoco:** Coordinator of the study, contributed to the design of the study, financial support, data analysis and the writing and discussion of the manuscript.

## Conflict of Interests

The authors declare no conflict of interests.

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