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Original Article

Sodium butyrate induced regulatory bone marrow-derived dendritic cells through up-regulation of indoleamine 2,3-dioxygense

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Abstract: Dendritic cells (DCs) are potent antigen-presenting cells, and play an important role in immune rejection, thus suppression of DC maturation and activation become a useful strategy in overcoming immune rejection after organ transplantation. In this study, we explored a novel approach in inducing regulatory DCs by stimulation of bone marrow-derived dendritic cells (BMDCs) with sodium butyrate (NaB), a short chain fatty acid metabolite from bacteria. BMDCs were stimulated with 0.5, 0.75 and 1 mmol/L NaB for 24 and 48 hours. 1 μg/ml lipopolysaccharide (LPS) was added alone to induce DC maturation. The cells co-stimulated with 1 mmol/L NaB and 1 μg/ml LPS were used to determine whether NaB is able to suppress LPS-induced DC maturation. Our results indicated that NaB effectively suppressed DC maturation and promoted differentiation of type 2 regulatory DCs (DC2), in which CD80, CD83 and CD86 were greatly suppressed, accompanied with decreased release of pro-inflammatory cytokines IL-12 and IFN-γ, but increased release of anti-inflammatory cytokine IL-10. Further investigation revealed that NaB also suppressed toll-like receptor 3 and programmed death 1 ligand 2 (B7-DC), but promoted expression of indoleamine 2,3-dioxygense (IDO), suppressors of cytokine signaling 2 and 3 (SOCS2 and SOCS3) after NaB treatment for 24 and 48 hours. We conclude that NaB suppressed DC maturation possibly through up-regulation of intracellular regulatory molecules IDO, SOCS2 and SOCS3. This observation provided an important information for the role and underlying molecular mechanisms of NaB in DCs-mediated immune tolerance.

Keywords: Dendritic cell, sodium butyrate, indoleamine 2,3-dioxygense, suppressor of cytokine signaling, immune tolerance

Introduction

Organ transplantation is an effective therapeutic approach in the treatment of multiple organ dysfunction. However this strategy is limited by short-term allograft survival and occurrence of graft-versus-host disease, thus it is essential to induce immune tolerance to grafted tissues [1-3]. Dendritic cells (DCs) are potent cells in the activation of immune responses against grafted tissues via release of pro-inflammatory cytokines and activation of T lymphocytes. After allograft is transplanted into recipient, host immature DCs are activated through internalization and digestion of allograft antigen. MHCII and costimulatory molecules CD80, CD83 and CD86 are up-regulated in the mature type 1 DCs (DC1) that release high levels of pro-inflammatory cytokines such as IL-17, IFN-γ and IL-12. These released cytokines and mediators further activate and recruit downstream Th1 type T cells and cytotoxic CD8+ T cells into the engrafted tissues for detrimental immune attack [4, 5]. However, regulatory DCs have low expression of these co-stimulating molecules and release low amount of pro-inflammatory cytokines, but produce high amount of anti-inflammatory cytokines such as transforming growth factor-β (TGF-β) and IL-10, demonstrating an immature and regulatory phenotype DCs (DC2) [6-8]. Studies in vitro and in vivo confirmed that the regulatory DCs are potent in inducing peripheral T cell tolerance and suppression of immune responses [6, 8, 9].

Because the distinct role of two DCs phenotypes in vitro and in vivo, modification of DC phenotypes has emerged as a promising therapeutic strategy in the treatment of autoimmune diseases and prolonging graft survival [10-14]. It has been reported that DCs over-expressing Jagged-1 (JAG1) and treated with anti-CD40L monoclonal antibody (mAb) significantly prolonged cardiac allograft survival in mice and recipients that beneficial effects are accompanied with up-regulation of TGF-β and increased population of regulatory T cells (Treg) [6]. Recent studies have demonstrated that butyrate acid has anti-inflammatory role in vitro and in vivo [15]. Butyric acid is a short-chain fatty acids (SCFAs), a metabolite from anaerobic periodontopathic bacteria [16]. It was reported that naïve T cells and memory CD4+ T cells stimulated with sodium butyrate (NaB) induced T unresponsiveness in OT-II ovalbumin-specific naive CD4(+) T-cells and memory T cells [17]. The immune regulatory effects were also observed in monocytes, addition of NaB was able to enhance IL-10 and IL-4 secretion, but reduce IL-12 and interferon-γ in the peripheral blood mononuclear cells [15, 18]. However little is known about the underlying molecular mechanisms of NaB immune suppressive function. It is accepted that NaB exerts anti-inflammatory effects through suppressing activity of histone deacetylase (HDAC) [15]. Because HDAC is an epigenetic regulator of gene expression, critically involved in multiple target gene expressions by deacetylation of histone in promoter region of target genes [16, 19], thus modulation of HDAC activity significantly influences cell immune responses, cell apoptosis and proliferation [20]. Studies have shown that NaB treatment inhibit CD4(+) T cell proliferation in response to IL-2 stimulation and promoted antigen-specific T cell anergy [17]. In addition, NaB also influences NK cell lysis activity and other cell types [20]. However little is known about the effects of NaB on DCs and the underlying molecular mechanisms.

It is known that there is high expression of indoleamine 2,3-dioxygenase (IDO), suppressor of cytokine signaling (SOCS) in tolerogenic DCs [8, 10, 13, 21]. IDO is a tryptophan-degrading enzyme and is regulated by IFN-γ. The elevated IDO expression levels are negatively correlated to graft rejection after transplantation [13]. SOCS is negative regulator of JAK/STAT signaling, also negatively associated with DC maturation and activation [22, 23]. Some studies have shown that over-expression and knock-down of SOCS1 and SOCS3 gene expression in DCs have significant effects on affect DC differentiation in vitro. Over-expression of SOCS3 in vivo was able to suppress some autoimmune disease development through inducing immune suppressive DCs and T cell tolerance [8, 24, 25].

In addition to IDO, other molecules such as program death-1 ligand (B7-H1 and B7-DC) participate in induction of tolerogenic DCs and peripheral T cell tolerance [26-28]. However whether NaB induce tolerogenic DCs through by affecting these IDO, SOCS and PD-1 signaling pathways is still unknown. In this study, we stimulated bone marrow-derived DCs (BMDCs) with different concentration of NaB, then analyzed DC phenotypes and functions. As expected, NaB significantly induced regulatory DCs, in association with elevated regulatory molecules in the treated DCs, the results further confirmed the immune regulatory role of NaB and its potential application in organ transplantation and treatment of autoimmune diseases.

Material and methods

Cell culture and treatment

Human blood was collected from healthy donors. The peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque (Lymphoprep TM) and cultured in RPMI1640 complete medium. Non-adherent cells were removed after 2 hours, remaining adherent cells were maintained in RPMI-1640 medium supplied with 10% FBS (Huangzhou Shijiqing Biotech Co. China), 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mmol/L L-glutamine (Gibco Inc), 1000 U/ml granulocyte macrophage-colony stimulating factor (GM-CSF) and 500 U/ml interleukin-4 (IL-4). After 6 days, the immature DC were collected and divided into four groups for treatment, 5 samples per group. The cells in all groups were maintained under GM-CSF and IL-4. The untreated cells were set up as negative controls, or treated with 1 μg/ml LPS or different concentration of NaB (NaB) (Sigma). In combinational treatment group, the cells were treated with 1 μg/ml LPS and 1 mmol/L NaB. All treated cells were harvested at 24 h and 48 h after treatment.

Table 1. Human primers

IDO	5'-GGAACTGGAGGCACTGATTTAA-3'	5'CAATGGGTAATGACAGGAATGC-3'
SOCS1	5'-GACCTGAACTCGCACCTCCTA-3'	5'-CCCCTGGTTTGTGCAAAGATA-3'
SOCS2	5'-GAAGTCGCGTTTTATCAGAATGC-3'	5'-ACTGGAGCCTGGACCTTTGAC-3'
SOCS3	5'-TGTTGCCATCAATGACCCCTT-3'	5'-CTCCACGACGTACTCAGCG-3'
GAPDH	5'-TGTTGCCATCAATGACCCCTT-3'	5'-CTCCACGACGTACTCAGCG-3'

Indoleamine 2,3-dioxygense (IDO), Suppressors of cytokine signaling (SOCS), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Flow cytometry analysis

The treated cells were washed with PBS supplied with 3% FBS, and stained with indicated fluorescence conjugated antibodies against human CD80, CD83, CD86, CD11c, TLR-3, TLR-4, B7-H1 and B7-DC (Life technologies, Grand Island, NY). Species matched isotype antibody was used as a negative control. After 30 minutes incubation on ice, the stained cells were washed with PBS supplied with 3% FBS for 2 times and fixed by 4% paraformaldehyde for 10 min, then washed and suspended in 400 μl PBS for FACS analysis. Flow cytometric measurements were performed using BD FACS Calibur (BD Biosciences, San Jose, CA). All data was analyzed using FlowJo software, version 8.8.4 (Tree Star Inc.).

Cytokine measurement

The treated cell supernatants were collected and stored at -80°C. The IL-12p40, IFN-γ and IL-10 in the cell supernatants were measured by ELISA kit (Life Science & Technology). Reaction was developed by TMB (Fischer Scientific, Pittsburgh, PA). Optical density was measured on Wellscan MK3 spectrometer (Lab systems Dragon Co.) at 450 nm. Detection limits are 0.15-40 ng/ml for IL-10, 31.2-2000 pg/ml for IL-12p40 and 15.6-1000 pg/ml for IFN-γ.

Quantitative RT-PCR

Total RNA was extracted from harvested cells by Trizol reagent (Life technology, Grand Island, NY). The cDNA was synthesized from 1-5 g total RNA by RT reaction kit, according to the manufacturers' instructions (Takara Clontech, Mountain View, CA). Primers were synthesized for amplification of human IDO, SOCS-1, SOCS-2 and SOCS3 cDNA (Shanghai Yinqun Biotech Ltd.); their sequences were described in **Table 1**. GAPDH was used as an internal control. SYBR green PCR master mix (Applied Biosystems, Foster City, CA) was used for quantitative RT-PCR reaction. Quantitative PCR was performed on 7500 Fast Real-Time PCR System (Life technology, Grand Island, NY) under condition of 95°C 5 min, then 40 cycles (95°C for 15 sec; 60° for 60 sec). Data was analyzed using 7500 Fast Software v2.3 software, and presented as Ct of experimental mRNA, relative to internal control GAPDH mRNA.

Statistical analysis

All data was presented as means ± standard error and statistically analyzed by using SSPS12.0 software and one-way ANOVA with bonferroni post-hoc was used for multiple comparisons and student *t* test was used for single comparison. Differences were considered significant at $P<0.05$.

Results

NaB suppressed DC maturation

After bone marrow-derived stromal cells were stimulated by 1000 U/ml GM-CSF and 500 U/ml IL-4 for 6 days, we observed immature dendritic cell phenotypes with few of multiple cell dendrites and cytoplasm granules (data not shown). The immature DC phenotype was further identified by flow cytometry analysis, with over 95% CD11b+CD11c+ phenotype DCs, indicating a high purity of prepared DCs after 6 days of stimulation in vitro. The untreated DCs have relative low expression levels of mature markers such as CD80, CD83 and CD86 (71.43±9.09%, 34.49±8.96%, 28.72±10.83% respectively). After 1 μg/ml LPS stimulation for 24 hours, the expression levels of CD80, CD83 and CD86 were increased and further increased after 48 hours stimulation compared to the untreated group, up to 91.98±4.66%, 55.18±8.95% and 94.97±3.08% respectively at 48 hours after LPS stimulation (**Figure 1**, $P<0.05$), demonstrating a matured phenotypes of LPS stimulated DCs. However CD80, CD83 and CD86 expression levels were attenuated in DCs treated with NaB at a dose and time-dependent manner as compared to the untreated DCs, with CD80, CD83 and CD86 expression levels down to 12.79±8.67%, 1.52±0.73% and 21.25±10.20% respectively at 48 hours

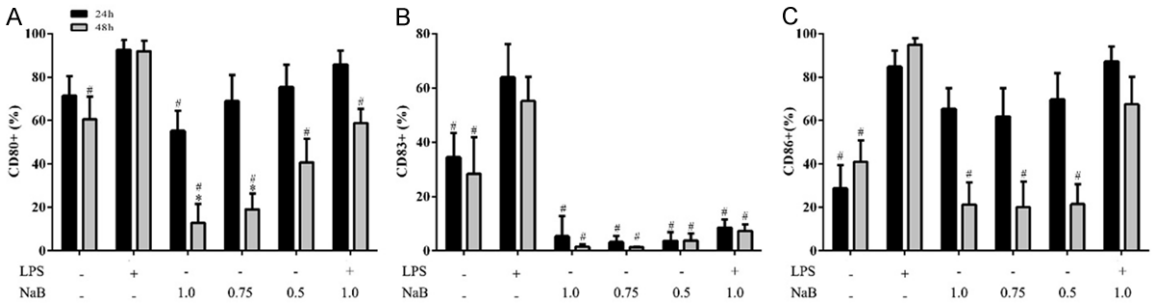

Figure 1. The sodium butyrate inhibited DC maturation. The peripheral blood mononuclear cells (PBMC) were respectively treated with 1, 0.75 and 0.5 mmol/L sodium butyrate (NaB). The cells treated with 1 μg/ml LPS or untreated were used as controls. In co-treatment group, the cells were treated with both 1 mmol/L NaB and 1 μg/ml LPS. After 24 and 48 hours of treatment, the expression of CD80, CD83, CD86 was measured by flow cytometry analysis. Data were presented as mean percentage of positive cells of CD11c+CD11b+ dentritic cells (DCs) ± standard error, n=5. *P<0.05, **P<0.01, compared to the untreated control, #P<0.05, ##P<0.1, 5 compared to the LPS treated.

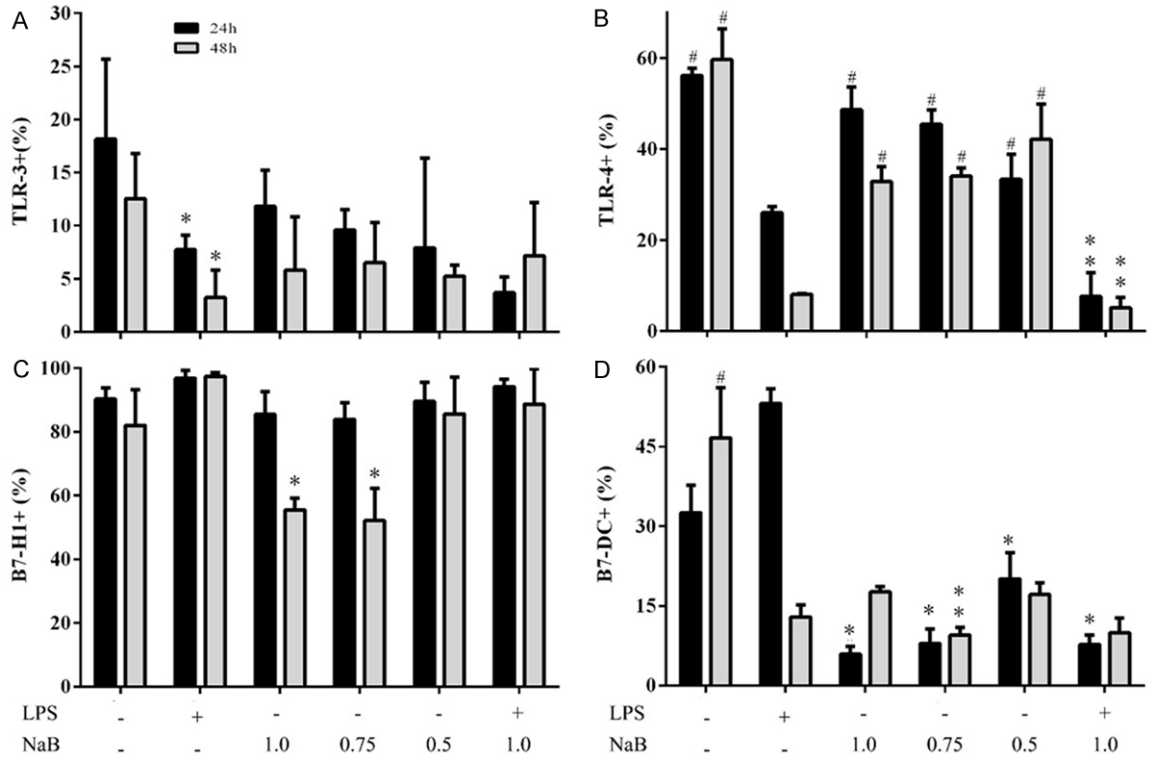

Figure 2. The sodium butyrate inhibited B7-H1, B7, TLR-3 and TLR-4 expression on DCs. After 24 and 48 hours of treatment, the expression of TLR3, TLR4, B7-H1 and B7-DC was measured by flow cytometry analysis. Data were presented as mean percentage of positive DCs ± standard error, n=5. *P<0.05, **P<0.01, compared to the untreated control, #P<0.05, ##P<0.1, 5 compared to the LPS treated.

after 1 mmol/L NaB treatment (**Figure 1**, P<0.05). In addition, we also observed the decreased CD80 and CD83 expression on DCs at 24 and 48 hours after co-treatment with 1 μg/ml LPS and 1 mmol/L NaB, as compared to the cells treated with 1 μg/ml LPS alone (**Figure 1**, P<0.05). However CD86 was not suppressed at 24 hours, but moderately suppressed at 48 hours by NaB treatment (**Figure 1**, P>0.05). The results indicated that NaB was able to suppress DC maturation under LPS stimulation, and induced more population of immune regulatory DC2 phenotype. Thus NaB may have potential to suppress immune responses via

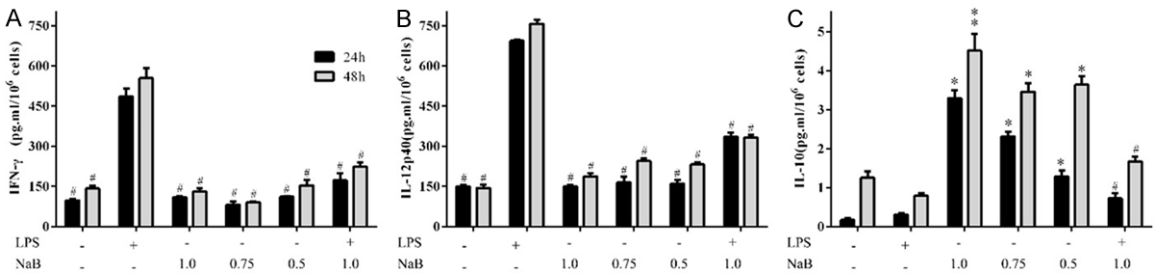

Figure 3. The sodium butyrate suppressed IFN-γ and IL-12p40 but enhanced IL-10 release from DCs. After 24 and 48 hours of treatment, the cell supernatants were collected and the expression levels of IFN-γ, IL-12p40 and IL-10 were measured by ELISA assay. Data were presented as mean pg.ml/10 (6) cells ± standard error, n=5. *P<0.05, **P<0.01, compared to the untreated control, #P<0.05, ##P<0.1, 5 compared to the LPS treated.

modulation of DC maturation and could be a useful adjuvant for increasing graft survival after graft transplantation.

NaB inhibited TLR3, TLR4, B7-H1 and B7-DC on DCs

TLR3, TLR4, B7-H1 and B7-DC are cell surface molecules expressed on DCs, the signaling pathways affect DC function and T cell activation. Virus-derived double-strand RNA and lipopolysaccharide (LPS) induce variety of downstream gene expression such as IL-12 and IFN-β expression through TLR3 and TLR4 signaling [29, 30]. B7-H1 (PD-L1) and B7-DC (PD-L2) induce negatively regulators of immune responses [28]. To further investigate whether NaB suppressed DC maturation is associated with down regulated TLR3, TLR4, B7-H1 and B7-DC on the treated DCs, we analyzed TLR3, TLR4, B7-H1 and B7-DC expression by flow cytometry analysis. As a result, we found that the cells treated with 1 μg/ml LPS expressed significant lower TLR3 and TLR4 at 24 hours after treatment as compared to the untreated cells (**Figure 2A** and **2B**, P<0.05). The further suppression was observed in the cells at 48 hours after LPS treatment, with 4-fold suppression of TLR3 and 7-fold suppression of TLR4 (**Figure 2A** and **2B**, P<0.05). In addition, NaB treatment also attenuated TLR3 and TLR4 expression at 24 and 48 hours, but the suppressive effects are less potent than LPS, and maximal suppression was observed in the cells treated with 0.5 mmol/L NaB instead of 1 mmol/L NaB. Co-treatment of DCs with both 1 μg/ml LPS and 1 mmol/L NaB resulted in additive suppression of TLR3 and TLR4 expression. The suppression of TLR4 was more than TLR3, with 8-10 fold decreases as compared to the untreated cells at 48 hours after treatment (**Figure 2A** and **2B**, P<0.01).

However, we did not observe significant changes for B7-H1 in the cells at 24 hour after treatment either with LPS and NaB alone or both. There was decreased expression of B7-H1 at 48 hours after treatment with 0.75 and 1 mmol/L NaB (P<0.05). In contrast with B7-H1, we observed largely suppressed B7-DC expression in the cells treated with LPS and NaB alone or both. The greatest suppression was observed in the cells treated with 0.75 mmol/L NaB from 46.65±9.44% in the untreated cells to 9.50±1.44% at 48 hours (P<0.01). Due to the marked suppression of B7-DC, there was no significant differences between the cells treated with NaB alone and combined treatment with LPS and NaB (**Figure 2C** and **2D**). The results indicated that 0.75 mmol/L NaB was an optimal concentration in the suppression of DC activation in vitro, that was correlated to the greatest suppression of DC maturation at this concentration (**Figure 1**).

NaB inhibited IFN-γ and IL-12p40, but enhanced IL-10 release from DCs

To investigate whether NaB suppression of DC maturation and activation is associated with alteration of cytokine release, we measure cytokine levels of IFN-γ, IL-12p40 and IL-10, in the supernatants of the treated cells. As expected, we observed the marked increase of IFN-γ and IL-12p40 in the cells after LPS treatment. Addition with NaB did not significantly affect the cytokine production. However, combinational treatment with both LPS and NaB significantly reversed IFN-γ and IL-12p40 upregulation for more than 2-3 fold than the LPS treated cells alone at 24 and 48 hours after treatment (P<0.05) (**Figure 3A** and **3B**), further indicating the immune suppressive effects of NaB. In contrast, IL-10, a potent anti-inflammatory cytokine was significantly elevated in the

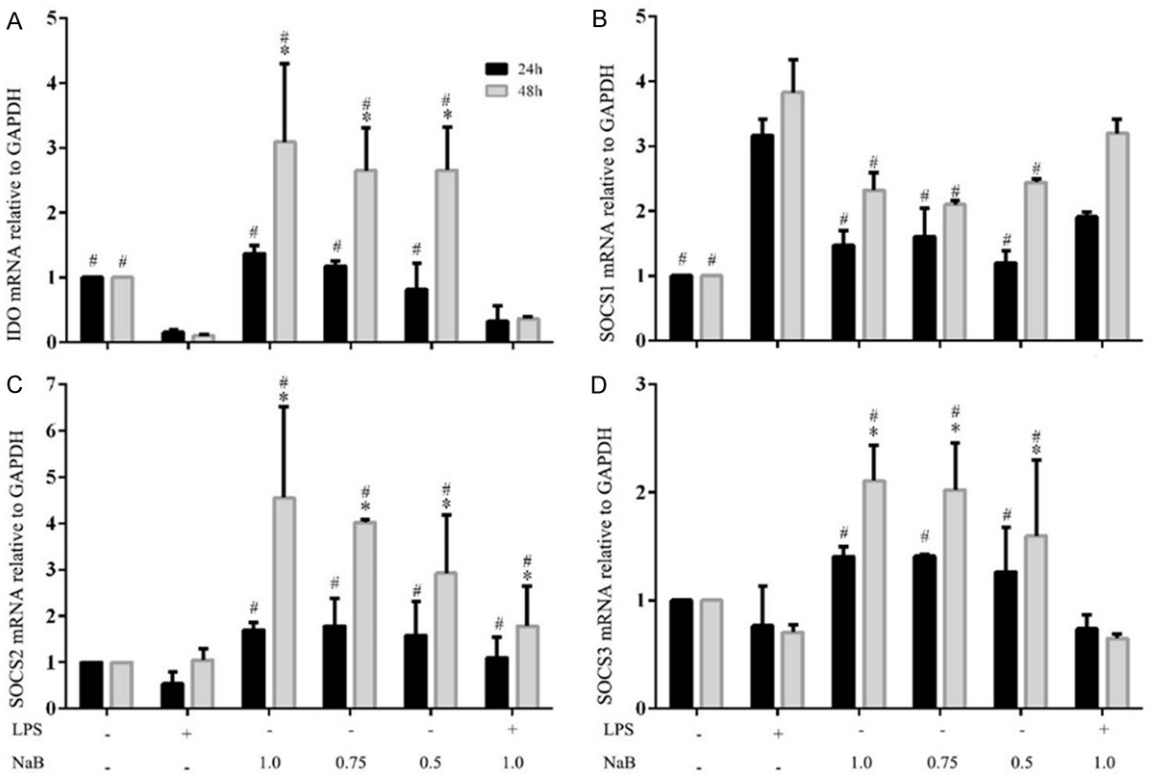

Figure 4. The sodium butyrate enhanced IDO, SOCS1, SOCS2 and SOCS3 mRNA in DCs. After 24 and 48 hours of treatment, the cells were collected for total RNA extraction. mRNA levels of IDO, SOCS1, SOCS2 and SOCS3 were analyzed by quantitative RT-PCR. GAPDH was used as internal control during qRT-PCR reaction. Data were normalized to internal control GAPDH and presented as mean DDCt relative to GAPDH ± standard error, n=5. *P<0.05, **P<0.01, compared to the untreated control, #P<0.05, ##P<0.1, 5 compared to the LPS treated.

NaB treated cells in a time and concentration dependent manner, with the greatest elevation in the cells treated with 1 mmol/L NaB for 48 hours (P<0.01) (**Figure 3C**). In contrast, IL-10 was moderately suppressed by LPS treatment and that was moderately reversed by co-treatment with LPS and NaB. The results further confirmed the important anti-inflammatory role of NaB through regulatory DC biased differentiation.

NaB enhanced IDO, SOCS2 and SOCS3 mRNA in DCs

Extensive studies showed that IDO and SOCS participate in the immune regulation and their expression levels positively reflect their involvement in immune suppression. However whether NaB induced immune suppression in association with their expression levels in DCs is still unknown. To define the effects of NaB on IDO and SOCS expression, we analyzed their mRNA levels in the treated cells by qRT-PCR assay. The results revealed that LPS treatment significantly suppressed IDO mRNA, whereas NaB elevated IDO mRNA levels at a time and concentration-dependent manner, and 3-fold increased IDO mRNA was observed in the cells at 48 hours after 1 mmol/L NaB treatment (**Figure 4A**). However suppressed IDO by LPS treatment was mildly reversed by NaB co-treatment. Similar to the effect on IDO, the expression levels of SOCS-1, SOCS-2 and SOCS-3 mRNA were also up-regulated by NaB treatment at a time and concentration-dependent manner. The greatest up-regulation of SOCS2 and SOCS3 was observed in the cells treated with 1 mmol/L NaB for 48 hours, and increased for at least 4-fold and 2-fold respectively (P<0.05) (**Figure 4C** and **4D**). LPS slightly affected SOCS2 and SOCS3 mRNA expression, but suppressed NaB-induced up-regulation, implying LPS may induce DC maturation through negative regulation of SOCS2 and SOCS3 expression. In contrast, SOCS1 mRNA was up-regulated by LPS treatment (P<0.05). However SOCS1 was not additively elevated by LPS and NaB co-treatment (**Figure 4B**). The different effect of LPS on

SOCS1 from SOCS2 and SOCS3 will be further studied in the further.

Discussion

In this study, we for the first time investigated the role of NaB in DC maturation and some target gene expression possibly relevant to DC maturation. Our results revealed that NaB treatment significantly suppressed DC maturation in vitro. CD80, CD83 and CD86 expression were suppressed on NaB-treated DCs. The maturation suppressed DC released a low amount of pro-inflammatory cytokines (IFN-γ and IL-12), but higher amount of anti-inflammatory cytokine IL-10. Therefore we conclude that NaB induced immune tolerance possibly through inducing regulatory DCs. However, the underlying mechanisms were not very clear. Our further investigation for the first time revealed that IDO, B7-H1, B7-DC, SOCS-2 and SOCS-3 might be involved in the NaB-induced regulatory DCs.

The immune regulatory role of NaB was reported previously in blood peripheral monocytes in vitro and mouse model with ulcerative colitis (UC) in vivo [18, 31]. It is also reported that NaB can improve mucosa lesion through suppressing inflammation in intestinal mucosa [17, 32]. Therefore, NaB is a potential agent in the treatment of autoimmune diseases and organ transplantation. However there is limited understanding the molecular basis of NaB in inducing immune tolerance and role in DCs differentiation. It is reported that NaB is able to suppress histone deacetylases (HDAC) activity [17]. HDAC modulates deacetylation of histone and induces multiple target gene transcription that is associated with cell proliferation, division and survival. It is reported that suppression of HADC activity lead to T cells apoptosis and T cell anergy [16, 19]. Although we did not measured HDAC activity, we observed that DC maturation was suppressed in the NaB-treated cells, consistent with the role of HDAC inhibitor in immune tolerance. In addition, we also observed attenuated signaling of TLR3 and B7-DC in the NaB-treated DCs. As important mediators in DC function, TLR3 and B7-DC expression levels significantly affect DC maturation and function [12, 27]. TLR3 and B7-DC signaling improve DC maturation and activation, whereas the suppressed signaling negatively affects DC maturation, function and survival [12, 23, 28, 33, 34]. In this study, we also found that the TLR3 and B7-DC were more suppressed than TLR4 and B7-H1 respectively in the NaB-treated DCs cells, suggesting that TLR3 and B7-DC may play more dominant role than TLR4 and B7-H1 in suppressing DC maturation.

Multiple cytokines and intracellular molecules interplay to control DC differentiation and function. IL-10 is considered a potent mediator in induction of regulatory DCs, the effect is thought mediated by activation of JAK/STAT3 signaling pathway [12, 23, 35]. The elevated IL-10 expression in the NaB-treated DCs induced regulatory DCs phenotype through regulating multiple downstream target gene expression. In addition, we also observed elevated IDO, SOCS2 and SOCS3 in the NaB-treated cells. These intracellular molecules are known critically involved in the induction of tolerogenic DCs and T cells [8, 13, 25, 36, 37]. We conclude that NaB may induce regulatory DCs through up-regulation of IDO, SOCS2 and SOCS3 signaling pathways. However we are still not clear whether the up-regulated gene transcription was induced by IL-10 or other mediators. It is possible that IL-10 is induced by up-regulated IDO, SOCS2 and SCOS3 expression levels in the NaB-treated DCs, because these intracellular molecules are potent regulatory DC regulators, up-regulation of these molecules promotesregulatory DC biased differentiation and subsequently release high amount of IL-10 from the NaB-treated DCs.

Taken together, our results revealed an immune modulatory property of NaB in DCs. The immune suppressive function of NaB may be mediated by increased expression of intracellular molecules IDO, SOCS2 and SOCS3. Thus study provide molecular basis of NaB in the induction of immune tolerance, and warrant us further investigation of signaling pathways of NaB in the regulation of IDO and other relevant gene expression.

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Disclosure of conflict of interest

None.

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