RESEARCH ARTICLE

2-(3-(chloromethyl)benzoyloxy)benzoic Acid Increases CD4$^+$ Regulatory T-Cell Population and FoxP3 Expression in Lipopolysaccharide-induced Mice

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Abstract

BACKGROUND: Lipopolysaccharide (LPS) has been reported to increase CD4$^+$ regulatory T-cell (CD4$^+$ Treg) populations. Acetylsalicylic acid (ASA) has been reported to have immunomodulatory activity, but it may induce chronic gastric ulceration. Another salicylic acid-bearing compound, 2-(3-(chloromethyl) benzoyloxy)benzoic acid (3-CH_2Cl), has been reported to have less gastric mucosal damage. However, the effect of 3-CH_2Cl on CD4$^+$ Tregs in LPS-induced mice is still unknown. Therefore, the present study was conducted to investigate the immunomodulatory effect of 3-CH_2Cl on CD4$^+$ T-cell and CD4$^+$ Treg populations as well as FoxP3 expression in LPS-induced mice.

METHODS: Synthesis of 3-CH_2Cl was performed by mixing salicylic acid and chloromethylbenzoylchloride with the catalyzation of pyridine, acetone and heat. The 3-CH_2Cl tablets were prepared using direct compression method. After intraperitoneal injection of 1 mg/kg BW LPS to mice, 60 mg/kg BW ASA or 60 mg/kg BW 3-CH_2Cl was given orally for 3 days. The splenocyte was obtained through splenectomy and collagenase digestion. The population of CD4$^+$ T-cells and CD4$^+$ Tregs, as well as the splenic FoxP3 expression were determined using flow cytometry technique.

RESULTS: CD4$^+$ T-cell populations in mice treated with LPS and 3-CH_2Cl or ASA were lower than those treated with LPS merely. Meanwhile, CD4$^+$ Treg populations and FoxP3 expression levels in mice treated with LPS and 3-CH_2Cl or ASA were higher than those treated with LPS merely.

CONCLUSION: Since 3-CH_2Cl could decrease CD4$^+$ T-cell population and increase CD4$^+$ Treg population mediated by the increase of FoxP3 expression in LPS-induced inflammation, it may act as a potential therapeutic drug to reduce inflammatory conditions.

KEYWORDS: 2-(3-(chloromethyl)benzoyloxy)benzoic acid, acetylsalicylic acid, CD4, T-regulatory cells, FoxP3, LPS

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Introduction

$CD3^+CD4^+CD25^+FoxP3^+$ regulatory T-cells ($CD4^+$ Tregs), are a subpopulation of T-cells with $CD3^+CD4^+C25^+FoxP3^+$ phenotype. Forkhead box-P3 (FoxP3) is a transcription factor commonly used in fluorescence-labeled flow cytometry as a key marker to detect the $CD4^+$ Treg populations.(1,2) $CD4^+$ Tregs are extensively studied due to its involvement in the pathogenesis and therapy of various autoimmune diseases, cancers and allergies, transplantation tolerance, and tissue engineering.(3-7) During inflammation, $CD4^+$ Tregs maintain the immune homeostasis by promoting the anti-inflammatory mechanism and decreasing pro-inflammatory T-cell subpopulations, including $CD4^+$ T, effector T-helper-1 (Th1) and T-helper 17 (Th17) cells.(8,9) In non-lethal acute inflammation mice models, lipopolysaccharide (LPS) could increase $CD4^+$ Treg populations (10), and suppress inflammatory components, such as $CD4^+$ T-cells, as well as pro-inflammatory cytokines.(8,11)

Acetylsalicylic acid (ASA) is one of non-steroidal anti-inflammatory drugs (NSAIDs) commonly prescribed due to its ability to inhibit the synthesis of proinflammatory prostaglandin (PG), specifically PGE2 by blocking cyclooxygenase (COX) at inflammation sites.(12,13) Treatment of 60 mg/kg ASA in untreated as well as experimental autoimmune encephalomyelitis mice for three days could successfully increase $CD4^+$ Tregs, which might be beneficial for attenuating the autoimmune responses. (12,14) Furthermore, ASA could inhibit graft rejection by augmenting $CD4^+$ Treg populations.(15,16) However, the effect of ASA on $CD4^+$ Tregs in LPS-induced mice is still unknown. It can be hypothesized that ASA treatment in LPS-induced mice might have a synergistic effect in increasing the $CD4^+$ Treg population and decreasing the $CD4^+$ T-cell population as a form of NSAID-mediated immunomodulation during inflammation. However, ASA may disrupt the cellular layer integrity through COX-1 inhibitory activity in gastric mucosal cells, thereby inducing chronic gastric ulceration.(17,18) A novel salicylic acid-bearing compound, namely 2-(3-(chloromethyl) benzoyloxy)benzoic acid (3-CH_2Cl), has numerous beneficial advantages over ASA and less gastric mucosal damage.(19-22) This compound could act as a better analgesic, antipyretic, anti-inflammatory, and antiplatelet activity to substitute ASA, hypothetically due to a higher binding affinity to anti-inflammatory COX-2 than ASA.(20) However, molecular mechanism of 3-CH_2Cl in alleviating the inflammatory response is still poorly understood.

Interestingly, a significant depletion of peripheral lymphocytes was reported in normal or LPS-induced mice treated with 3-CH_2Cl.(21) The potency of 3-CH_2Cl to alleviate graft rejection and autoimmune disease by increasing the $CD4^+$ Treg population has not been explored. Therefore, the present study was conducted to investigate the immunomodulatory effect of 3-CH_2Cl on $CD4^+$ T-cell and $CD4^+$ Treg populations, as well as FoxP3 expression in LPS-induced mice.

Methods

Synthesis of 3-CH_2Cl

Synthesis of 2-(3-(chloromethyl)benzoyloxy)benzoic acid was performed according to the previously reported methods. (20) In brief, 1.8 mmol salicylic acid (Brataco, Surabaya, Indonesia), 7.2 mmol 3-chloromethylbenzoylchloride (Sigma-Aldrich, St. Louis, MO, USA), 1.7 x 10^{-6} mmol pyridine (Merck, Darmstadt, Germany), and 1.48 x 10^{-6} mmol acetone (Merck) were mixed homogeneously. The reaction was then generated using a Millstone Organic Synthesis Unit microwave (MicroSYNTH, Göttingen, Germany) for 1 minute, 600 W. To detect the presence of salicylic acid, thin layer chromatography was performed using ferric chloride ($FeCl_3$), silica gel F254 (Merck) as stationary phase, and hexane-ethanol (1:2 v/v) as mobile phase.

Preparation of 3-CH_2Cl Tablet

The 3-CH_2Cl tablet was prepared according to the previously reported methods (22) using direct compression method. Three hundred mg 3-CH_2Cl was homogenized with 9.38% Neusilin (Gangwal Chemicals, Mumbai, India). Further, the mixture was added with 0.92% sodium lauryl sulfate (Sigma-Aldrich), 2.33% croscarmellose sodium (FMC Biopolymer, Philadelphia, USA), 5% microcrystalline cellulose (Gujarat Microwax, Gujarat, India), and spray dried lactose (Foremost Farms, Pasig, Philippines) and centrifuged for 2 minutes at 100 rpm. The resulting tablet (total weight of 800 mg) was compressed into tablet forms with a single punch machine (Jenn Chian Machinery, Feng-Yuan, Taiwan). The hardness, friability, disintegration time, and drug dissolution parameters of the tablets were then evaluated.

LPS-induced Mice Model and Treatment Groups

Sixteen Swiss-webster mice (8-week-old, 20-30 g, male) were purchased from the Veterinary Farma Center,

Surabaya, Indonesia. The experimental animals were maintained based on the Statement of Ethical Eligibility (No.: 001/EC-FKH/Eks/2022). The mice were housed in a temperature-controlled room (20-24°C) with 65% relative humidity and 12-hour light/dark cycle. The mice were allowed to consume food and water *ad libitum*. The mice were then randomly allocated into four groups (4 animals/group), namely the untreated, LPS-only, LPS+3-CH_2Cl, and LPS+ASA groups.(12) To induce inflammation, the mice in the LPS-only, LPS+3-CH_2Cl, and LPS+ASA groups were administered intraperitoneally with a single dose (1 mg/kg BW) of LPS from *Escherichia coli* O111:B4 (Sigma-Aldrich).(12) Meanwhile, mice in the untreated group were injected with phosphate-buffered saline (PBS) (Genaxxon Bioscience, Ulm, Germany) only. ASA (Bayer, Leverkusen, Germany) or 3-CH_2Cl were given orally to the mice with a single dose of 60 mg/kg BW, respectively. The untreated and LPS-only groups were given 3% Pulvis Gummi Arabicum (Brataco). ASA and 3-CH_2Cl administrations were repeated twice a day, one hour after LPS injection and six hours after the first dose. The treatments were repeated for three consecutive days. At the end of the experiment (12), the animals were euthanized using 90 mg/kgBW ketamine and 10 mg/kgBW xylazine to isolate the spleen.

Splenocytes Isolation and Preparation

Splenocytes isolation was performed according to the previously optimized protocol.(23) The spleen was incubated for 30 minutes with a buffer containing 100 U/mL collagenase type IV (Worthington, Lakewood, NJ, USA), 0.2% DNAse (Worthington), and RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA). The resulting cell suspensions were filtered through 70 µm cell strainer (Biologix, Shandong, China). To lyse the red blood cells, ammonium-chloride-potassium buffer was added to the cell suspensions. After 2 minutes incubation on ice, the splenocytes were centrifuged for 5 minutes at 1,500 rpm, and the cell pellet was washed with 0.5% bovine serum albumin (BSA) in PBS. The pellet was then resuspended in 0.5% BSA in PBS. To adjust the splenocyte concentration until it reached 5 x 10^6 cells/mL, the splenocytes were stained with 0.5% trypan blue (Contacare Ophthalmics and Diagnostics, Vadodara, India) and counted under a light microscope (100x magnification).

Detection of $CD4^+$ Tregs with Antibody Panels

For extracellular staining, splenocytes (5 x 10^6 cells/mL) were incubated with 10 µg of FITC Anti-Mouse CD25 Antibody (Elabscience, Houston, TX, USA) for 20 minutes in the dark. The splenocytes were then incubated with the same amount of PE Anti-Mouse CD3 Antibody (Elabscience) and PerCP/Cyanine5.5 Anti-Mouse CD4 Antibody (Elabscience) for another 20 minutes in the dark. The splenocytes were washed with 0.5% BSA in PBS, centrifuged for 5 minutes at 1,800 rpm, and the supernatant was discarded. For intracellular staining, splenocytes were resuspended with eBioscience Permeabilization Buffer (Thermo Fisher Scientific) and incubated for 10 minutes in the dark. The splenocytes were washed twice and resuspended in 0.5% BSA in PBS. Ten µg of APC Anti-Mouse Foxp3 Antibody (Elabscience) was added to the splenocytes. After further washing and the addition of permeabilization buffer, the splenocytes were fixed with BD Cytofix fixation buffer (Becton Dickinson, Franklin Lakes, NJ, USA), and analyzed with flow cytometry (23) using a FACSCalibur flow cytometry (Becton Dickinson) and Flowing Software (Turku Bioscience, Turku, Finland). For all flow cytometry experiments, the data from 10,000 events was acquired. Firstly, the area with the densest cell population was determined. Population of $CD4^+$ T-cells was then selected by entering the CD3 and CD4 parameters. The dot plot was divided into four quadrants. The $CD4^+$ T-cells population was shown in the upper right position. After that, $CD4^+$ Treg populations were determined from the $CD4^+$ T-cell population by entering CD25 and FoxP3 parameters. The dot plot formed was divided into four quadrants and the population of $CD4^+$ Tregs was shown in the upper right position (Supplementary 1).

Statistical Analysis

One-way ANOVA with Tukey post hoc test were used for multiple comparisons. To compare mean differences in particular groups, unpaired t-test was used. A $p<0.05$ were considered significant. All data were presented as the mean±standard deviation (SD). The data were analyzed and graphically presented using GraphPad Prism Software v.8.0.2 (Boston, MA, USA).

Results

ASA and 3-CH_2Cl Decreased $CD4^+$ T-Cell Population in LPS-induced Mice

$CD4^+$ T-cell count of the LPS-only, LPS+ASA, and LPS+3-CH_2Cl groups were significantly higher compared with that of the untreated group (p=0.001, p=0.0011, and p=0.0017, respectively). $CD4^+$ T-cell count was not significantly different between the LPS-only and LPS+ASA as well

as LPS+3-CH_2Cl groups (Figure 1A). Representative $CD4^+$ T-cell percentage (%$CD4^+$) compared with the total splenocyte in each group was presented in dot plot cytogram (Figure 1B). The %$CD4^+$ of the LPS-only, LPS+ASA, and LPS+3-CH_2Cl groups were significantly higher compared with that of the untreated group (p=0.0004, p=0.0047, and p=0.0028, respectively). Furthermore, the %$CD4^+$ of the LPS+ASA group was lower than that of the LPS-only group, although no significant difference was observed between these groups (p=0.0746). Meanwhile, the %$CD4^+$ of the LPS+3-CH_2Cl group was significantly lower than that of the LPS-only group (p=0.0222). In addition, the %$CD4^+$ of the LPS+ASA group was higher than that of the LPS+3-CH_2Cl group, although no significant difference was observed between these groups (p=0.6052) (Figure 1C).

ASA and 3-CH_2Cl Increased $CD4^+$ Treg Population in LPS-induced Mice

$CD4^+$ Treg count of the LPS-only group was significantly higher compared with that of the untreated group (p=0.0135). $CD4^+$ Treg count of the LPS+ASA and LPS+3-CH_2Cl groups were significantly higher than that of LPS-only group (p=0.0001 and p=0.0022, respectively) (Figure 2A). Representative $CD4^+$ Treg percentage (%$CD4^+$ Treg) compared with the total $CD4^+$ T-cells in each group was shown in dot plot cytogram (Figure 2B). The %$CD4^+$ Treg of the LPS-only, LPS+ASA, and LPS+3-CH_2Cl groups were significantly higher than that of untreated group (p=0.0011, p=0.0002, and p<0.00001, respectively). Even though the %$CD4^+$ Treg of the LPS+ASA and LPS+3-CH_2Cl groups were higher, no significant differences were observed between these two groups and the LPS-only groups (Figure 2C).

ASA and 3-CH_2Cl Increased FoxP3 Expression in LPS-induced Mice

To further investigate the role of ASA and 3-CH_2Cl on the expression of FoxP3, histogram analysis and mean fluorescence intensity (MFI) measurement of splenocytes labelled with FoxP3 antibody were performed (Figure 3A). The MFI value of the LPS-only, LPS+ASA, and LPS+3-CH_2Cl groups were significantly higher than the one of the untreated group (p=0.0044, p<0.0001, and p<0.0001, respectively), indicating an increase in FoxP3 expression. The FoxP3 expression of LPS+ASA and LPS+3-CH_2Cl groups was significantly higher compared with the one of the LPS-only group (p=0.0098 and p=0.0005, respectively). In addition, 3-CH_2Cl increased the FoxP3 expression ~15% higher than ASA (p=0.0378).

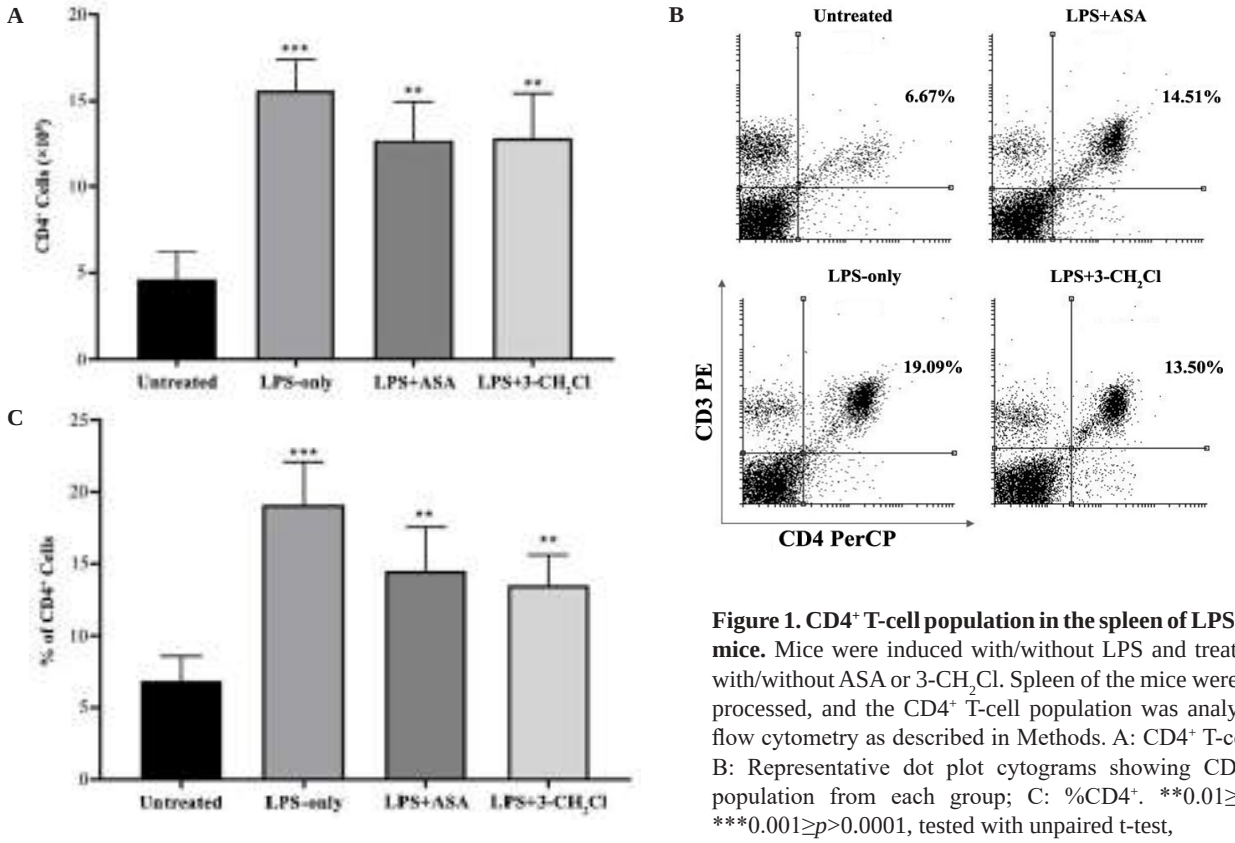

Figure 1. $CD4^+$ T-cell population in the spleen of LPS-induced mice. Mice were induced with/without LPS and treated orally with/without ASA or 3-CH_2Cl. Spleen of the mice were isolated, processed, and the $CD4^+$ T-cell population was analyzed with flow cytometry as described in Methods. A: $CD4^+$ T-cell count; B: Representative dot plot cytograms showing $CD4^+$ T-cell population from each group; C: %$CD4^+$. **0.01≥p>0.001; ***0.001≥p>0.0001, tested with unpaired t-test,

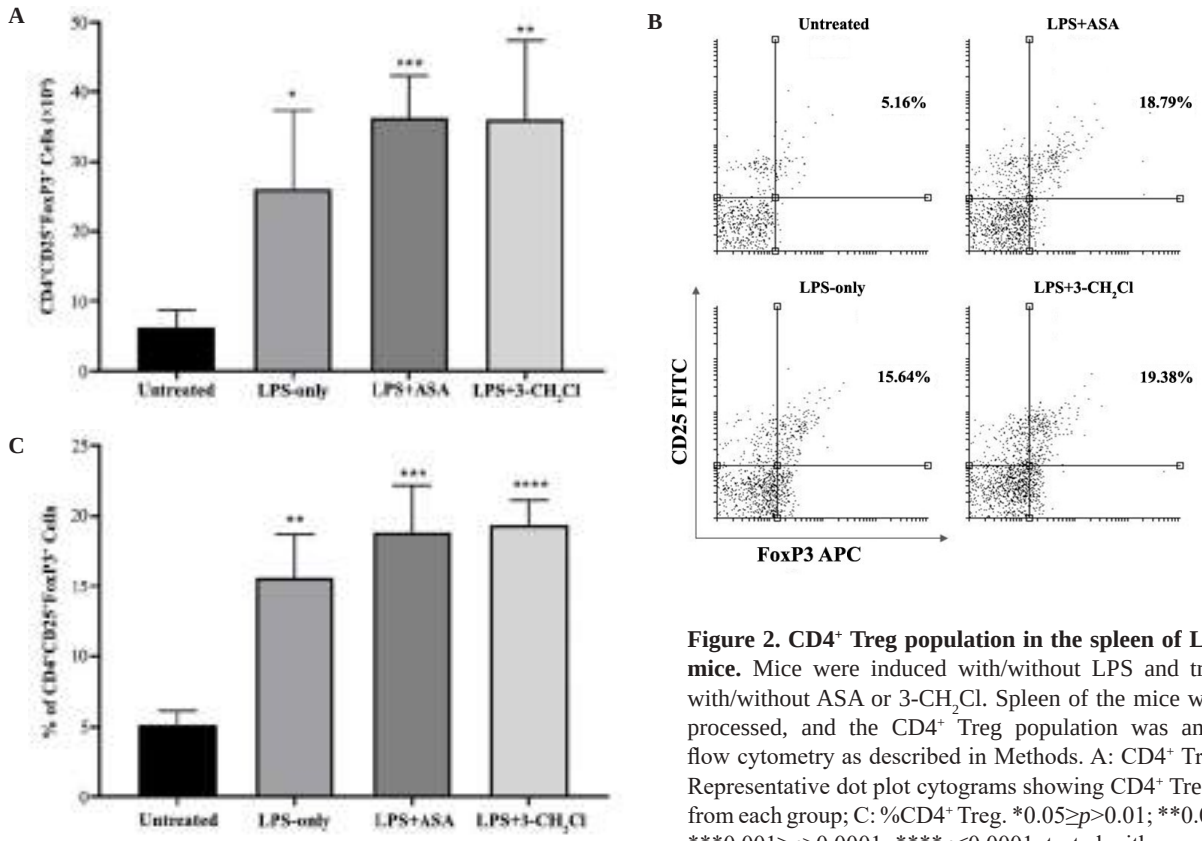

Figure 2. CD4⁺ Treg population in the spleen of LPS-induced mice. Mice were induced with/without LPS and treated orally with/without ASA or 3-CH_2Cl. Spleen of the mice were isolated, processed, and the CD4⁺ Treg population was analyzed with flow cytometry as described in Methods. A: CD4⁺ Treg count; B: Representative dot plot cytograms showing CD4⁺ Treg population from each group; C: %CD4⁺ Treg. *0.05≥*p*>0.01; **0.01≥*p*>0.001; ***0.001≥*p*>0.0001; ****$p \leq 0.0001$, tested with unpaired t-test.

Discussion

CD4⁺ Tregs provide a new insight on immune response regulation, particularly in inflammation, autoimmune diseases, and transplantation immunology, mainly on preventing and/or delaying graft rejection. During inflammation, pro-inflammatory antigens, such as LPS can activate the adaptive immune responses through the induction of pro-inflammatory cells, such as CD4⁺ T-cell and CD4⁺ Treg population.(8,10,11) In the present study, there was a significant increase in splenic CD4⁺T-cells count and percentage in all LPS-treated groups. Oral treatment of ASA and 3-CH_2Cl did not affect CD4⁺ T-cell count in the spleen of LPS-induced mice. As expected, ASA as well as 3-CH_2Cl could decrease the %CD4⁺ in the spleen of LPS-induced mice. Therefore, anti-inflammatory function of ASA and 3-CH_2Cl on the adaptive immunity is mediated by suppression of CD4⁺T cells.(12)

Interestingly, the results of the present study demonstrated the synergistic effect of ASA in its therapeutic dose range and LPS in its non-lethal dose, which enhanced CD4⁺ Treg count and percentage in LPS-induced mice. These results strengthen the hypothesis that ASA could potentially make hosts more susceptible to tolerance induction.(12,15) Even though the direct effect of 3-CH_2Cl in the adaptive immune system, particularly in T-cells is still understudied, the increase of splenic CD4⁺ Tregs mediated by 3-CH_2Cl during the inflammatory condition would be theoretically beneficial for patients with autoimmune diseases or allografts.(14) Furthermore, 3-CH_2Cl has been reported to have lesser gastric erosion (20), making this compound even more potential as a better candidate for treating autoimmune diseases and transplant patients. To support this statement, a further clinical study should be conducted.

CD4⁺ Tregs has been known to respond to natural (for example: pathogenic bacteria) or inducible inflammatory conditions (for example: LPS) by increasing their suppressive function (10,24), which further prevents the activation of pro-inflammatory components.(11) FoxP3 expression is the key factor for CD4⁺ Tregs immunomodulation. The results of the present study showed that salicylic acid bearing compounds, such as ASA or 3-CH_2Cl, could significantly increase splenic FoxP3 expression in inflammatory condition regardless of the cell type. Inflammatory cytokines such as IL-1β, IL-6 and TNF have been reported to antagonize FoxP3 expression and restrict CD4+ Tregs function.(6,7)

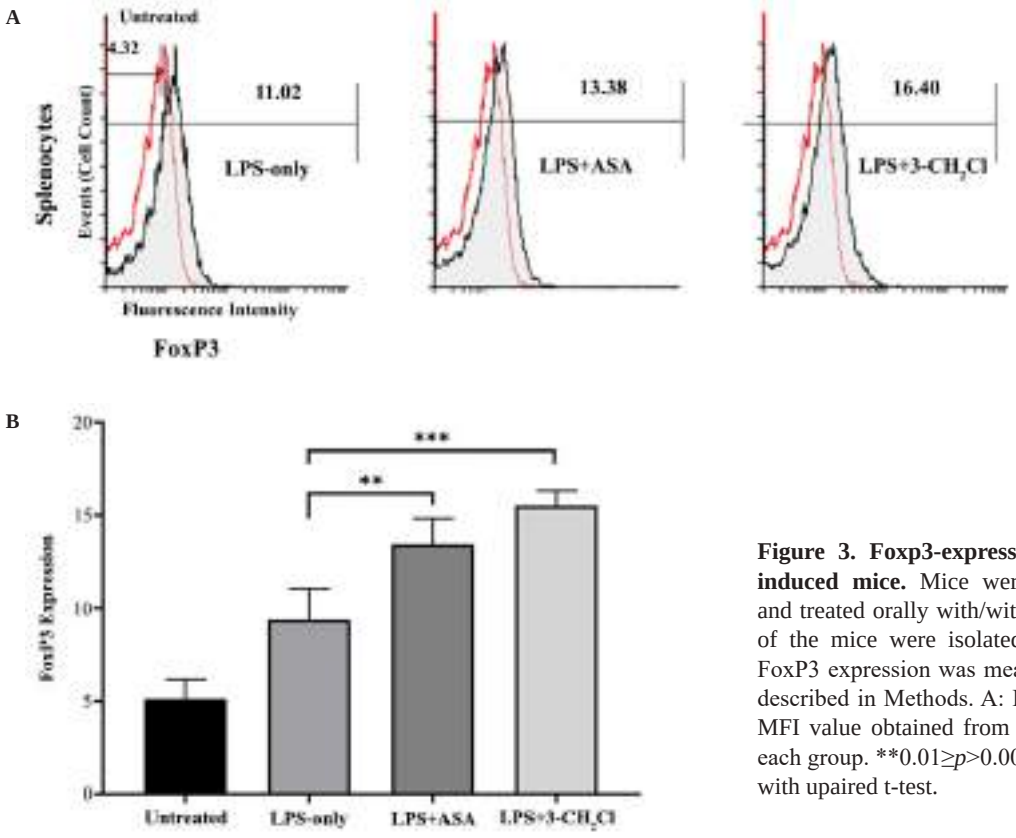

Figure 3. Foxp3-expression in splenocytes of LPS-induced mice. Mice were induced with/without LPS and treated orally with/without ASA or 3-CH_2Cl. Spleen of the mice were isolated, processed, and the splenic FoxP3 expression was measured with flow cytometry as described in Methods. A: Representative histogram with MFI value obtained from each group. B: MFI value of each group. **$0.01 \geq p > 0.001$; ***$0.001 \geq p > 0.0001$, tested with upaired t-test.

Since ASA and 3-CH_2Cl have also been reported to repress the secretion of proinflammatory cytokines (15,16,21), those compounds might increase FoxP3 expression through the suppression of proinflammatory cytokines. These findings open a new insight on the anti-inflammatory mechanism of those compounds in adaptive immunity to suppress the proinflammatory environment, mainly through increasing $CD4^+$ Treg population and positively regulating FoxP3 expression.

Several *in vitro* studies using human cell lines revealed the function of COX, particularly COX-2 as the positive regulator for $CD4^+$ Treg.(8,25) Therefore, the increase of Treg after LPS induction might be triggered by the typical augmentation of LPS-induced COX-2 expression in innate as well as adaptive immune cells.(26,27) ASA, 3-CH_2Cl and many other salicylic acid-bearing compounds are well known for their COX-inhibitory property. However, it seems that $CD4^+$ Treg production increases after COX-2 inhibition with those compounds. ASA and 3-CH_2Cl increase $CD4^+$ Treg population possibly through stimulation by several anti-inflammatory signaling components produced during COX-2 inhibition, such as IL-10 or TGF-β.(10,28)

Many anti-inflammatory drugs were developed before the discovery of Treg. The present study may bring forward the neglected mechanism of salicylic acid-bearing anti-inflammatory drugs, especially 3-CH_2Cl and ASA, in increasing Treg population, which might be beneficial for the optimization of their clinical application, such as effective dose adjustment or development of more potent drug combinations for the therapy of several inflammatory-linked immune disorders, such as autoimmunity (15), graft rejection (29) and diabetes (30).

Besides $CD4^+$ Tregs, $CD8^+$ T-cell subsets carrying regulatory properties have been discovered and called "$CD8^+$ Tregs".(31) $CD8^+$ Tregs can be induced in various allograft conditions.(32) $CD8^+$ Treg could stimulate $CD4^+$ Treg production (33,34) and compensate their function (35), and is believed as another master regulator in autoimmunity and solid organ transplantation. Therefore, further studies are needed to better understand the potential implication of ASA and 3-CH_2Cl on $CD8^+$ Tregs population.

Conclusion

Taken together, 3-CH_2Cl could decrease $CD4^+$ T-cell population and increase $CD4^+$ Treg population mediated by the increase of FoxP3 expression in LPS-induced inflammation. 3-CH_2Cl may act as a potential therapeutic drug to reduce inflammatory conditions.

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Authors Contribution

YT, CC, KF, HI and HW were involved in concepting and planning the research. YT, CC, SK, SE and HW performed the methodology. NJ, OC, IM, WH, YR and SY performed the data acquisition/collection. YT, CC, JN, DA, DL and HW calculated the experimental data, performed the analysis, and interpreting the results. NJ, OC, and IM designed the figures. All authors took parts in manuscript preparation and giving critical revision of the manuscript.

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