**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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Received date: May 29, 2023; Revised date: Aug 1, 2023; Accepted date: Aug 7, 2023

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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-CH₂Cl), has been reported to have less gastric mucosal damage. However, the effect of 3-CH₂Cl on CD4+ Tregs in LPS-induced mice is still unknown. Therefore, the present study was conducted to investigate the immunomodulatory effect of 3-CH₂Cl on CD4+ T-cell and CD4+ Treg populations as well as FoxP3 expression in LPS-induced mice.

**METHODS:** Synthesis of 3-CH₂Cl was performed by mixing salicylic acid and chloromethylbenzoylchloride with the catalyzyation of pyridine, acetone and heat. The 3-CH₂Cl 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-CH₂Cl 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-CH₂Cl 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-CH₂Cl or ASA were higher than those treated with LPS merely.

**CONCLUSION:** Since 3-CH₂Cl 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

Introduction

CD4+CD25+FoxP3+ regulatory T-cells (CD4+ Tregs), are a subpopulation of T-cells with CD3+CD4+CD25+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-CH2Cl), 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-CH2Cl 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-CH2Cl.(21) The potency of 3-CH2Cl 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-CH2Cl on CD4+ T-cell and CD4+ Treg populations, as well as FoxP3 expression in LPS-induced mice.

Methods

Synthesis of 3-CH2Cl

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 10⁶ mmol pyridine (Merck, Darmstadt, Germany), and 1.48 x 10⁶ 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 (FeCl3), silica gel F254 (Merk) as stationary phase, and hexane-ethanol (1:2 v/v) as mobile phase.

Preparation of 3-CH2Cl Tablet

The 3-CH2Cl tablet was prepared according to the previously reported methods (22) using direct compression method. Three hundred mg 3-CH2Cl 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,
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. 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 population of CD4+ T-cells was shown in the upper right position. After that, the CD4+ Treg populations were determined 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$_2$Cl Decreased CD4+ T-Cell Population in LPS-induced Mice

CD4+ T-cell count of the LPS-only, LPS+ASA, and LPS+3-CH$_2$Cl 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-CH₂Cl 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-CH₂Cl 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-CH₂Cl 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-CH₂Cl group, although no significant difference was observed between these groups ($p=0.6052$) (Figure 1C).

ASA and 3-CH₂Cl 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-CH₂Cl 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-CH₂Cl 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-CH₂Cl groups were higher, no significant differences were observed between these two groups and the LPS-only groups (Figure 2C).

ASA and 3-CH₂Cl Increased FoxP3 Expression in LPS-induced Mice

To further investigate the role of ASA and 3-CH₂Cl 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-CH₂Cl 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-CH₂Cl groups was significantly higher compared with the one of the LPS-only group ($p=0.0098$ and $p=0.0005$, respectively). In addition, 3-CH₂Cl increased the FoxP3 expression ~15% higher than ASA ($p=0.0378$).
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-CH2Cl did not affect CD4+ T-cell count in the spleen of LPS-induced mice. As expected, ASA as well as 3-CH2Cl could decrease the %CD4+ in the spleen of LPS-induced mice. Therefore, anti-inflammatory function of ASA and 3-CH2Cl 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-CH2Cl in the adaptive immune system, particularly in T-cells is still understudied, the increase of splenic CD4+ Tregs mediated by 3-CH2Cl during the inflammatory condition would be theoretically beneficial for patients with autoimmune diseases or allografts.(14) Furthermore, 3-CH2Cl 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-CH2Cl, 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)
Since ASA and 3-CH₂Cl 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-CH₂Cl 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-CH₂Cl 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-CH₂Cl 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-CH₂Cl on CD8⁺ Tregs population.

**Conclusion**

Taken together, 3-CH₂Cl could decrease CD4⁺ T-cell population and increase CD4⁺ Treg population mediated by the increase of FoxP3 expression in LPS-induced inflammation. 3-CH₂Cl may act as a potential therapeutic drug to reduce inflammatory conditions.
Acknowledgments

We would like to thank RSUD Dr. Soetomo Surabaya for providing the research facilities and Research and Community Service Institute (LPPM) Widya Mandala Catholic University Surabaya, who has funded this research. The research was funded by Research and Service Institute Widya Mandala Surabaya Catholic University (Grant No. 7240/WM01/N/2022) to Wuryanto Hadinugroho.

Authors Contribution

YT, CC, KF, HI and HW were involved in concep ting 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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DOI: 10.18585/inabj.v15i4.2429