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Title: Inflammatory kidney injury in trichloroethylene hypersensitivity syndrome mice: possible role of C3a receptor in the accumulation of Th17 phenotype

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Abstract: Trichloroethylene (TCE) is a common organic solvent which can cause TCE hypersensitivity syndrome (THS) in exposure workers. THS is an adverse skin disorder with severe inflammatory kidney damage. Complement C3a receptor (C3aR) acts as a specific receptor for the key complement cleavage product C3a and involves multiple inflammatory responses, but the role of C3aR in TCE induced kidney inflammatory injury remains unknown. In this study, BALB/c mouse model of skin sensitization induced by TCE was set up in the presence or absence of C3aR antagonist (C3aRA). Kidney pathology and renal function, expression of inflammatory mediators and C3aR, changes in Th17 cell numbers, and activation of signal transducer and activator of transcription 3 (STAT3) in the kidney were examined. TCE sensitization produced histopathological and functional damage to the kidney, accompanied by increased levels of interleukin (IL-) 1 β , IL-6, and IL-23. Local accumulation of Th17 cells and enhanced phosphorylation of STAT3 were also seen in the impaired kidney in TCE sensitization-positive mice. C3aR was mainly located in the impaired glomerulus and upregulated in TCE sensitization-positive mice. C3aRA pretreatment alleviated the structural and functional kidney damage and the inflammatory cytokine and Th17 responses by TCE sensitization, and specifically reduced the phosphorylation of STAT3. Together, our results demonstrate that C3aR signaling promotes the inflammatory responses and regulates the accumulation of Th17 phenotype via phosphorylation of STAT3 in TCE sensitization induced inflammatory kidney damage. C3aR may serve as a potential therapeutic target in TCE sensitization mediated kidney injury.

Highlights

TCE sensitization induced structural and functional disorders of renal damage.

Inflammatory cytokines IL-1 β , IL-6, and IL-23 were evaluated in kidney by TCE sensitization.

C3aR-antagonist pretreatment alleviated expression of inflammatory cytokines in impaired kidney.

C3aR blockade suppressed STAT3 pathway and decreased accumulation of Th17 cells.

1 **Inflammatory kidney injury in trichloroethylene hypersensitivity syndrome mice:**
2 **possible role of C3a receptor in the accumulation of Th17 phenotype**

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20 **Declarations of interest**

21 None.

ABSTRACT

Trichloroethylene (TCE) is a common organic solvent which can cause TCE hypersensitivity syndrome (THS) in exposure workers. THS is an adverse skin disorder with severe inflammatory kidney damage. Complement C3a receptor (C3aR) acts as a specific receptor for the key complement cleavage product C3a and involves multiple inflammatory responses, but the role of C3aR in TCE induced kidney inflammatory injury remains unknown. In this study, BALB/c mouse model of skin sensitization induced by TCE was set up in the presence or absence of C3aR antagonist (C3aRA). Kidney pathology and renal function, expression of inflammatory mediators and C3aR, changes in Th17 cell numbers, and activation of signal transducer and activator of transcription 3 (STAT3) in the kidney were examined. TCE sensitization produced histopathological and functional damage to the kidney, accompanied by increased levels of interleukin (IL-) 1 β , IL-6, and IL-23. Local accumulation of Th17 cells and enhanced phosphorylation of STAT3 were also seen in the impaired kidney in TCE sensitization-positive mice. C3aR was mainly located in the impaired glomerulus and upregulated in TCE sensitization-positive mice. C3aRA pretreatment alleviated the structural and functional kidney damage and the inflammatory cytokine and Th17 responses by TCE sensitization, and specifically reduced the phosphorylation of STAT3. Together, our results demonstrate that C3aR signaling promotes the inflammatory responses and regulates the accumulation of Th17 phenotype via phosphorylation of STAT3 in TCE sensitization induced inflammatory kidney damage. C3aR may serve as a potential therapeutic target in

44 TCE sensitization mediated kidney injury.

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46 **Key words:** Trichloroethylene; Hypersensitivity; Kidney; C3a receptor; Th17

47

48 **Abbreviations:**

49 TCE, trichloroethylene

50 THS, trichloroethylene hypersensitivity syndrome

51 ODMLT, Occupational Dermatitis Medicamentosa-like of Trichloroethylene

52 C3aR, complement C3a receptor

53 C3aRA, C3aR antagonist

54 STAT3, signal transducer and activator of transcription 3

55 EM, erythema multiforme

56 ED, exfoliative dermatitis

57 SJS, Stevens-Johnson syndrome

58 TEN, toxic epidermal necrolysis

59 CP, classical pathway

60 LP, lectin pathway

61 AP, alternative pathway

62 TNF- α , tumor necrosis factor α

63 BUN, blood urea nitrogen

64 Cre, creatinine

65

1. Introduction

Trichloroethylene (TCE) is a common organic solvent widely used in some Asian countries, particularly in China (Li et al., 2019). TCE can cause TCE hypersensitivity syndrome (THS) mainly via inhalation and skin-contact in occupational workers. THS is also called Occupational Dermatitis Medicamentosa-like of Trichloroethylene (ODMLT) in China, which is an adverse skin disorder characterized by skin rashes, mucosal damage, high fever, liver and kidney dysfunction, and superficial lymphadenopathy (Huang et al., 2014a; Kamijima et al., 2013; Nakajima et al., 2018). The THS spectrum ranges from a mild type such as erythema multiforme (EM) to severe forms such as exfoliative dermatitis (ED), Stevens-Johnson syndrome (SJS), and toxic epidermal necrolysis (TEN) (Kamijima et al., 2013; Watanabe, 2011). Although the incidence of THS is low, the mortality can be up to 9-13% (Dai et al., 2015; Kamijima et al., 2007).

The pathogenesis of THS remains unclear, which limits the effective clinical treatment. Studies show that THS is an occupational immune disease, in which delayed allergic reaction (type IV hypersensitivity) underlies the skin lesion (Ordaz et al., 2017; Xueqin et al., 2018). However, type IV hypersensitivity theory fails to explain why severe liver and kidney dysfunction is concomitant in most THS patients. According to recent studies, cytotoxic type (type II) and immune-complex type (type III) allergic reactions may also be involved in THS, supported by higher serum levels of complement C3 and immunoglobulin G in THS cases (Zhao et al., 2012).

As an important player in type II and III allergic reaction, complement cascade activate to work as a defender in innate immunity via three major pathways, classical pathway (CP), lectin pathway (LP), and alternative pathway (AP) (De Vriese et al., 2015). All pathways lead to the central component C3 to be cleaved by C3 convertase to generate the bioactive fragments C3a and C3b. C3a is a vital inflammatory mediator by binding to the specific G protein-coupled receptor C3aR. C3aR is expressed on cell membranes in many cell types, such as epithelial cells, macrophages, T lymphocytes and dendritic cells (Reichhardt and Meri, 2018). Recent studies show that C3aR signaling is involved in various renal inflammatory dysfunctions, including diabetic kidney disease (Li et al., 2015), atypical hemolytic uremic syndrome (Sartain et al., 2018), focal segmental glomerulosclerosis (Liu et al., 2017) and immunoglobulin A nephropathy (Zhang et al., 2017).

Multiple visceral organ damage is a serious complication of THS, and hepatitis is the most common and fatal (Kamijima et al., 2007; Xu et al., 2009). Kidney injury is another important form of organ damage in THS patients. It occurs later than hepatitis but can seriously affect the prognosis and quality of the patient's life. (Liu, 2009; Wei et al., 2008). B-ultrasound examination of THS cases shows an enlarged kidney and thickened renal cortex in an early stage while reduced volume and thinned cortex in the advanced stage, with diffuse inflammation in both kidneys (Liu, 2009; Zhang et al., 2011). Furthermore, there are also positive urinary protein, and up-regulated serum levels of urea nitrogen (BUN) and creatinine (Cre) (Liu, 2009; Xu et al., 2009; Zhang et al., 2011). Thus, the kidney lesion in THS involves both structural damage

and functional disorders, in which local inflammatory response plays a vital role. Consistently, our previous studies have also found that pathological changes in the kidney and impairment of renal function in TCE sensitized mice (Yang et al., 2018). However, whether and how C3aR signaling participates in TCE-induced renal injury remains unknown.

Therefore, this study aims to explore the possible role of C3aR signaling in renal injury using a mouse model of skin sensitization induced THS with a specific C3aR antagonist.

2. Material and methods

2.1 Mice

Female BALB/c mice (6-8weeks old, n=50) were obtained from the Experimental Animal Center of Anhui and maintained in Anhui Medical University. Mice were housed in the specific pathogen-free environment with food and water *ad libitum*. The housing conditions were maintained at $22.5 \pm 0.5^{\circ}\text{C}$, $50 \pm 5\%$ humidity, and 12h light/dark cycle. After a week for adaptation, mice were randomly assigned to blank control, vehicle control, TCE treatment group, and C3aR antagonist (C3aRA, SB290157, Calbiochem) pre-treatment group. All experimental protocols were approved by the Experimental Animal Ethics Committee of Anhui Medical University (No. LLSC20160312). The care and use of mice

132 complied with NIH guide and the 3R principle.

134 2.2 Experimental design

136 The mouse model of skin sensitization induced by TCE was conducted according
137 to our previously established method (Wang et al., 2015; Zhang et al., 2018). Briefly,
138 for TCE treatment group, 100 μ l 50% TCE, diluted by olive oil and acetone (TCE:
139 olive: acetone = 5: 2: 3, v/v/v), and the equal volume freund's complete adjuvant
140 (FCA) were subcutaneously injected to each mouse for the first sensitization. Next,
141 100 μ l 50% TCE was applied on mouse skin for the continued sensitization on Days 4,
142 7, and 10, respectively. Finally, 100 μ l 30% TCE (TCE: olive: acetone = 3: 2: 5, v/v/v)
143 was applied on the same skin area for the challenge at Days 17 and 19. For C3aRA
144 pre-treatment group, a single dose of 10mg/kg C3aRA was intraperitoneally injected
145 30 minutes before the challenge, and the other procedures were the same as the TCE
146 treatment group. For the vehicle control group, the same proportion of olive and
147 acetone was given to each mouse at the same time point. For the blank control group,
148 only saline was used as a substitute (Fig. 1).

149 The scores of mouse skin allergy were assessed as follows: 0 point, for entirely
150 normal; 1 point, for scattered redness or mild swelling; 2 points, for moderate
151 swelling or diffuse redness; 3 points, for intensive erythema or swelling. Score $\geq$ 1 was
152 defined as a positive sensitization and the rate of sensitization was calculated.
153 According to the allergy scores, mice in the TCE treatment group were further divided

into TCE positive sensitization sub-group (TCE+ group), TCE negative sensitization sub-group (TCE- group). Mice in TCE with C3aRA pretreatment group were divided into TCE sensitization positive with C3aRA pre-treatment sub-group (C3aRA/TCE+ group), and TCE sensitization negative with C3aRA pre-treatment sub-group (C3aRA/TCE- group). Three days after challenge, all mice were euthanized by CO₂ in the current study to collect blood and kidneys in the bacteria-free environment for the following measurements. Specifically, H&E staining, immunoturbidimetric assay, and ELISA were performed on all mice. Immunohistochemistry, immunofluorescence, qRT-PCR, and western blot assay were carried out in all mice with pathological kidney injury in TCE+ (n=7) and C3aRA/TCE+ groups (n=5) and in some mice (randomized selection, n=5-7) in TCE- and C3aRA/TCE- groups.

2.3 H&E staining

The kidney samples fixed in 4% paraformaldehyde for 2 days, were embedded in paraffin after the tissue being dehydrated, cleared with increasing concentrations of ethanol. 5µm thick sections were then cut onto glass slides for H&E staining following the standard protocols with hematoxylin staining for nucleus and eosin staining for cytoplasm. The slides were visualized and analyzed under an optical microscope (Olympus BX53, Japan).

2.4 Immunoturbidimetric assay

176

177 Renal function indicators, BUN and Cre, were determined by sarcosine oxidase
178 and urease method kit (Nanjing Jiancheng Bioengineering Institute, China). Serum
179 samples were analyzed following the instruction manual and read in Microplate
180 Reader (Bio-Tek, μ Quant) at 640nm for BUN or 546nm for Cre.

181

182 2.5 ELISA

183

184 Mouse serum was obtained to detect the level of inflammatory cytokine
185 interleukin (IL)-6 via ELISA commercial kit (Elabscience, Wuhan, China). The
186 standard series and equal volumes of samples were added into the 96-well plates
187 which were pre-attached with specific IL-6 antibodies. The value of optical density
188 (OD) was determined by the microplate reader (BioTek μ Quant) and the sample
189 level of IL-6 was obtained according to the standard curve.

190

191 2.6 Immunohistochemistry and immunofluorescence

192

193 The paraffin sections of kidney tissue were used for immunohistochemical DAB
194 staining. After the routine dewaxation in xylene and hydration in ethanol gradients
195 (100% ethanol, 95% ethanol, and 75% ethanol, v/v), the sections were washed with
196 PBS and treated with 3% H_2O_2 to eliminate the activity of endogenous peroxidase.
197 Then the sections were heated in 0.01 M sodium citrate buffer for antigen retrieval,

with non-specific binding being blocked by 10% normal goat serum in PBS for 30 minutes at 37°C. Next, anti-IL-1 β antibody (Abcam, ab9722, UK), anti-IL-6 antibody (Abcam, ab208113, UK), or anti-IL-23 antibody (Abcam, ab45420, UK) were added onto the sections at 4°C overnight. Thereafter, the sections were incubated with biotin-labeled secondary antibody (ZSJQ-BIO, China) after being taken out from a refrigerator to warm up to the room temperature. Following on, the horseradish enzyme labeled oval chain enzyme was added to the sections. Finally, a DAB kit (ZSJQ-BIO, China) and hematoxylin were used to stain the target protein and nucleus respectively.

For immunofluorescence, frozen kidney samples embedded in OCT were sectioned at 4 μ m in a freezing microtome (Leica, CM1950, Germany). The sections were fixed in pre-cold acetone for 5 minutes and then washed with PBS for 3 times with 5 minutes each time. The sections were blocked by goat serum for 2 hours at 37°C before being incubated with anti-C3aR rabbit antibody (Santa, sc-53785, USA), or co-incubation with anti-CD4 (Abcam, ab25804, UK) and anti-IL-17A (Abcam, ab79056, UK) antibodies. The fluorescein labelled secondary antibody was added onto the sections for 2 hours, and then 4', 6-diamidino-2-phenylindole (DAPI, Merck, USA) was added to stain the nucleus. After wash with PBS for 3 times, the sections were examined and pictures taken under a fluorescence microscope (Olympus, IX73, Japan).

2.7 *qRT-PCR*

220

221 25mg of kidney tissue was taken out to isolate total RNA by TRIzol reagent
222 (Invitrogen, USA). The concentration and purity of the extracted RNA were
223 determined by NanoDrop One (Thermofisher, USA) and diluted to 0.5g/L by enzyme
224 free water. The cDNA was obtained by RevertAid First Strand cDNA Synthesis Kit
225 according to the manufacturer's manual. Then, using a SYBR Green I Master kit, PCR
226 amplification was carried out in a Light Cycler 480 system (Roche, Switzerland) as
227 the following program: 95°C for 10 minutes for initial denaturation, 45 cycles
228 consisting of 95°C for 15 seconds for denaturation, 60°C for 15 seconds to annealing
229 and 72°C for 20 seconds for extension. The following primer sequences were used for
230 qRT-PCR:

231 *C3aR*:

232 forward, 5'-TATCCCATCCATCATTATTC-3';

233 reverse, 5'-CAAAGGCTACCACCCAGA-3';

234 *GAPDH*:

235 forward, 5'-GGAGAGTGTTTCCTCGTCCC-3';

236 reverse, 5'-ATGAAGGGGTCGTTGATGGC-3'.

237

238 *2.8 Western blot*

239

240 25mg of kidney tissue was cut off to extract proteins in radio
241 immunoprecipitation lysis buffer (RIPA) containing protease and phosphatase

inhibitor. The protein concentration was measured by BCA assay and the final protein concentration adjusted to 10mg/ml mixed with loading buffer. Following the denature, 10µl sample was loaded on sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) for electrophoresis. Next, the proteins were transferred from the gels to PVDF membrane (0.45µm, Millipore, USA) with protein marker and blocked in BSA for 2 hours. Afterwards, anti-STAT3 (CST, 12640S, USA), anti-phospho-STAT3 (Tyr 705, CST, 9145S, USA), or GAPDH antibodies (CST, 5174S, USA) were both diluted in 1:1000 and incubated with the PVDF membrane at 4°C overnight. After being washed in TBST 3 times with 10 minutes each time, goat anti-rabbit IgG antibody diluted in TBST (1:20,000) was incubated with PVDF membrane. Finally, the chemiluminescence (Advansta, 171005-80, USA) was used to detect the membrane proteins in chemiluminescence system (Tanon, Fine-do X6, China).

2.9 Statistical Analysis

Data were presented as means $\pm$ SD and analyzed by SPSS13.0 software package (Chicago, USA). One-way analysis of variance (ANOVA) or two-way ANOVA, followed by Bonferroni test, was used to test interactions between the two factors and the differences between different groups. A *p-value* <0.05 was considered as significant difference in the present study.

3. Results

3.1 Sensitization rate

The sensitization rate of all mice was 40% (16/40) in the present study according to the skin reactions. In TCE treatment group, 9 mice out of 20 showed positive sensitization with the skin allergy scores ≥ 1 and the sensitization rate was 45%. In C3aRA pre-treatment group, 7 mice had skin allergy scores ≥ 1 , and the sensitization rate was 35%. The sensitization rate between TCE treatment group and C3aRA pre-treatment group was not statistically different ($P > 0.05$). The details of skin allergy scores are shown in Table 1.

3.2 Histological changes and dysfunction of kidney induced by TCE

H&E staining showed intact structures of both glomerulus and renal tubule with the orderly arrangement of kidney cells in blank control, vehicle control, TCE-, and C3aRA/TCE- group. In TCE+ group, glomerular cell proliferation, renal tubular cell swelling, and tubular morphological changes were found in 7 mice. These pathological changes were alleviated in 5 mice observed from C3aRA/TCE+ group. The renal dysfunction was also ameliorated by C3aRA pretreatment as evidenced by lower expression of BUN and Cre compared with that in C3aRA/TCE+ group. The representative graphs of H&E staining and results of BUN and Cre are presented in Fig. 2A-C and the incidence of kidney pathological injury is listed in Table 1. These

results show that C3aRA pretreatment improved structural and functional damage in the kidney due to TCE sensitization.

3.3 TCE induced inflammation status of the blood

IL-6 level in peripheral blood was determined to investigate the inflammation status induced by TCE sensitization. As shown in Fig. 2D, levels of IL-6 in TCE+ and C3aRA/TCE+ groups were both higher than TCE- and C3aRA/TCE- group, respectively ($P<0.05$). However, no statistical difference was found among the blank control group, the vehicle control group, TCE- group, and C3aRA/TCE- group ($P>0.05$). These data suggest that TCE sensitization caused a systemic inflammatory response in mice.

3.4 Expression of C3aR in impaired kidney with or without C3aRA pretreatment

Immunofluorescence results show that C3aR was mainly expressed on glomerulus in the damaged kidney in TCE+ group, while little expression of C3aR was observed in C3aRA/TCE+ group under the fluorescence microscope. Moreover, little expression of C3aR was found in the blank control group, the vehicle control group, and C3aRA/TCE- group (Fig. 3A-F). These results suggest that the dose and time of C3aRA (10mg/kg SB290157, intraperitoneal injected 30 minutes before challenge) was appropriate and effective for competitive inhibition of C3aR signaling

in this mouse model for investigating the downstream effects.

C3aR mRNA was calculated by the $2^{-\Delta\Delta Ct}$ method and calibrated with *gapdh*. No significant difference was found between blank control, vehicle control, TCE-, and C3aRA/TCE- group ($P>0.05$). *C3aR mRNA* level was statistically higher in TCE+ group than that in the vehicle control group ($P<0.05$). However, no statistical difference was found between C3aRA/TCE+ group and TCE+ group ($P>0.05$). Together, these data suggest that C3aRA can block the C3aR via direct binding to the receptor protein without effect on *C3aR mRNA* levels in TCE sensitization response (Fig. 3G).

3.5 *C3aRA pretreatment alleviated local over-expressed inflammatory cytokines in impaired kidney*

Inflammatory cytokines, including IL-1 β , IL-6, and IL-23 were evaluated by immunohistochemistry and quantified by Image J software (Version 1.52A, USA). No visible expressions of the above inflammatory mediators were seen in the blank control group, the vehicle control group, TCE- group, and C3aRA/TCE- group (Supplement data, sFig. 1). Data from the optical density quantification showed no significant changes in IL-1 β , IL-6, and IL-23 proteins in TCE- group and C3aRA/TCE- group, compared with the vehicle control group ($P>0.05$). However, IL-1 β , IL-6, and IL-23 proteins were significantly increased in TCE+ group compared with those in vehicle control and TCE- groups ($P<0.05$), while these increased IL-1 β ,

IL-6, and IL-23 proteins seen with TCE sensitization were attenuated by C3aRA (C3aRA/TCE+ group vs. TCE+ group: $P<0.05$) (Fig. 4). These data suggest that changes to these inflammatory mediators are TCE sensitization specific and pretreatment with C3aRA counteracts these changes.

3.6 C3aR signaling regulates local accumulation of Th17 cells in impaired kidney

Accumulation of Th17 cells in the kidney tissue was evaluated by immunofluorescence with double labeled with anti-CD4 and anti-IL-17A antibodies. The Th17 phenotype was identified by the overlap of CD4 and IL-17A fluorescence. As shown in Fig. 5A, massive accumulation of Th17 cells was presented in TCE+ group, while little overlap of CD4 and IL-17A was found in C3aRA/TCE+ group. Of note, quantification of CD4 and IL-17A levels by the average fluorescence intensity (Image J) show that IL-17 level in C3aRA/TCE+ group was lower than that in TCE+ group ($P<0.05$). Although no significant difference in CD4 levels between C3aRA/TCE+ group and TCE+ group, its levels were up-regulated CD4 levels in the above two groups when compared with those in blank control, vehicle control, TCE-, and C3aRA/TCE- groups. No visible overlap was found in the blank control group, vehicle control group, TCE- group, and C3aRA/TCE- group (Supplement data, sFig. 2), suggesting that Th17 increase is a response to TCE sensitization. Taken together, these data suggest that C3aR signaling may be required for Th17 differentiation in local inflammatory injury in the kidney.

3.7 C3aRA pretreatment suppressed STAT3 pathway in impaired kidney

Total signal transducer and activator of transcription 3 (t-STAT3) and phospho-STAT3 (p-STAT3) protein levels were determined by western blot (Fig. 6). The results of western blot bands for t-STAT3 and p-STAT3 show that there was no significant difference in intensity values among blank control, vehicle control, TCE- group, or C3aRA/TCE- group ($P>0.05$). However, intensity values of p-STAT3 were statistically higher in TCE+ group and C3aRA/TCE+ group compared to vehicle control group ($P<0.05$), while the intensity value in C3aRA/TCE+ group was lower than that in TCE+ group ($P<0.05$). This indicates that STAT3 is a downstream pathway for C3aR signaling in TCE sensitization.

4. Discussion

THS is a rare but life-threatening occupational health issue in Asia, different from other solvent-induced irritating contact dermatitis. The severe visceral complication, especially hepatitis and kidney damage, is a serious problem of THS (Liu, 2009). Complement activation may contribute to the visceral damage based from an association between decreased level of C3 and degree of liver damage in THS cases (Huang et al., 2014b). As another major target organ in THS is the kidney and complement activation in inflammatory kidney injury has drawn increasing attention

from Chinese scholars and doctors. Animal studies demonstrate C5aR signaling in damaged kidney, associated with the inflammatory response via phosphorylation of mitogen-activated protein kinase (MAPK) pathway to aggravate TCE-induced inflammatory kidney damage (Zhang et al., 2016). However, the role of another vital inflammatory mediator C3aR signaling is unknown in TCE-induced renal inflammatory injury.

To this end, we set up BALB/c mouse model of TCE sensitization and intraperitoneal injection with or without C3aRA pretreatment (SB290157, 10 mg/kg, 30 minutes before the challenge) based on our previous studies (Wang et al., 2014). SB290157 is effective and selective antagonist and can block the specific binding of C3a to C3aR without inhibiting DNA synthesis in cells, which has already been proven by studies from us and others (Hutamekalin et al., 2010; Ikeda et al., 2014; Mizutani et al., 2009; Wang et al., 2014). Besides, a single dose of 10mg/kg SB290157 via intraperitoneal injection does not cause kidney damage (Supplement data, sFig. 3). In this study, the negative immunofluorescence results of C3aR in C3aRA/TCE+ group also confirmed that C3aR signaling in the kidney was completely blocked by the applied dose and duration of application of SB290157. Remarkably, our data also show no change of *C3aR* mRNA in the kidney after SB290157 pretreatment. This is consistent with the fact that SB290157 is a competitive antagonist of C3a and acts on the receptor proteins without effect on its mRNA or DNA synthesis. The effectiveness of C3aR treatment in alleviating histological and functional kidney damages suggests that C3aR signaling is involved

in the kidney injury induced by TCE and blocking the C3aR signaling by the antagonist may be a protective measure in the kidney injury.

C3aR signaling is a G protein-coupled receptor which is widely expressed on immune and non-immune cells (Reichhardt and Meri, 2018). C3aR signaling regulates the secretion of various inflammatory cytokines and chemokines, which has already been shown in multiple autoimmune diseases (Hawksworth et al., 2017). Zhang et al found that C3aR deletion could reduce the secretion of pro-inflammatory factors, such as IL-1 β , IL-6, TNF- α , TGF- β , and ameliorated the kidney injury in mouse model of IgA nephropathy (Zhang et al., 2017). Li et al demonstrated that blockade of C3aR signaling by C3aR antagonist prevented endothelial-myofibroblast transition via inhibiting the wnt/ β -catenin pathway (Li et al., 2015). Notably, our immunofluorescence microscopy showed that C3aR was mainly expressed on glomerulus while little C3aR expression was observed in renal tubules in TCE sensitized mice. As glomerulus is the primary structure in the kidney for filtration and excretion function, we propose that up-expression of C3aR in glomerulus is at least one of the reasons for TCE induced histopathological changes and functional disorders of the kidney.

To explore the possible role of C3aR in TCE induced local kidney injury, we measured inflammatory cytokines required for Th17 differentiation including IL-1 β , IL-6, and IL-23 in the kidney (Acosta-Rodriguez et al., 2007). Studies have confirmed that IL-1 β , IL-6, and IL-23 are necessary for CD4⁺ T cells to differentiate into a Th17 phenotype, which has been shown to mediate inflammatory reactions and cellular

immune responses in systemic lupus erythematosus (Raymond et al., 2018), IgA nephropathy (Peng et al., 2013), and glomerulonephritis (Ifuku et al., 2013). In the present study, large amounts of IL-1 β , IL-6, and IL-23 were secreted in the damaged kidney from TCE sensitized mice, with consistent increase in Th17 phenotype in the kidney. However, C3aRA pretreatment down regulated the release of the above inflammatory mediators and the accumulation of Th17 phenotype. These data suggest that C3aR signaling is a key upstream pathway which links the secretion of IL-1 β , IL-6, and IL-23 to Th17 differentiation and the pro-inflammatory response in TCE-induced kidney injury.

The STAT3 pathway in the kidney tissue was examined to further explore the mechanisms of C3aR-driven Th17 cell proliferation. STAT3 is known to have a specific role for Th17 differentiation by acting as the crucial transcription factor both in vitro and in vivo (Kim et al., 2018; Ren et al., 2016; Vogel et al., 2015). STAT3 normally exists in an inactive cytoplasmic form, and once activated, translocates into the nucleus and is phosphorylated on tyrosine 705 (Tyr 705) to activate the transcription of a broad array of targeted genes (Rebe et al., 2013; Yoshimura et al., 2007). Recently, a study showed that phospho-STAT3 protein was up-regulated in cultured human T cells with TCE treatment, and this suggests that STAT3 pathway participates in T cell activation in THS (Jie et al., 2017). However, whether STAT3 pathway is involved in TCE induced kidney injury is unknown. Our western blot results show that phospho-STAT3 protein was upregulated in TCE sensitized mice and this increase was suppressed by C3aRA pretreatment. Together with the

up-regulated levels of IL-1 β , IL-6, and IL-23 in TCE sensitization induced kidney injury, the present findings highlight that STAT3 pathway is activated in the process of Th17 differentiation in renal inflammatory damage and can be suppressed by C3aRA pretreatment.

It is worth noting that crosstalk between glomerulus and renal tubules provides new insight into kidney inflammatory injury following TCE sensitization. As presented above, C3aR was mainly localized in the damaged glomerulus in TCE sensitization positive mice, while proinflammatory cytokines including IL-1 β , IL-6, and IL-23, were mostly expressed in impaired renal tubules. This points to a tubulo-glomerular interaction involved in kidney inflammatory response by TCE sensitization. The mechanisms for this interaction remain to be explored in the future.

In summary, we have demonstrated that C3aR signaling plays a critical role in the regulation of Th17 proliferation in TCE sensitization induced inflammatory kidney damage, via STAT3 activation and this can be alleviated by C3aRA pretreatment. Thus, C3aR signaling may serve as a possible therapeutic target for TCE sensitization induced renal inflammatory injury.

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462

463 **Declarations of interest**

464 None.

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 565

Figure Legends

Fig. 1. Flow diagram of the mouse model of skin sensitization induced by TCE.

Note: 50% TCE was diluted by olive oil and acetone (TCE: olive: acetone = 5: 3: 2, v/v/v); 30% TCE was also diluted by olive oil and acetone (TCE: olive: acetone = 3: 2: 5, v/v/v). Abbreviation: TCE, trichloroethylene; FCA, freund's complete adjuvant; s.c., subcutaneous injection; i.p., intraperitoneal injection.

Fig. 2. TCE induced histological changes with dysfunction in the kidney and inflammation status in the blood.

A) Histological changes in the kidney of blank control group (upper panel, left), vehicle control group (upper panel, middle), TCE positive sensitization sub-group (TCE+, upper panel, right), TCE negative sensitization sub-group (TCE-, lower panel, left), TCE positive sensitization with C3aRA pre-treatment sub-group (C3aRA/TCE+, lower panel, middle), and TCE negative sensitization with C3aRA pre-treatment sub-group (C3aRA/TCE-, lower panel, right). Glomerular cells proliferation, renal tubular cells swelling, and tubular morphological changes were found in TCE+ group (magnification, 400×). Scale bar indicated 50 μm. B) Serum levels of BUN. C) Serum levels of CRE in various groups. D) Serum levels of IL-6 in various group. Data showed that both renal function indicators (BUN and Cre) and inflammation index (IL-6) were up-regulated in TCE+ group and down-regulated by C3aRA pretreatment.

Note: Compared to vehicle group, ^a $P < 0.05$; Compared to relevant TCE- group,

^b $P < 0.05$; Compared to C3aRA/TCE+ group, ^c $P < 0.05$. The principal simple effect is

TCE treatment. There is interaction between TCE and C3aRA in BUN. The ANVOA table is shown in supplement data.

Fig. 3. Expression of C3aR in the local kidney with or without C3aRA pretreatment. A) Blank control group; B) Vehicle control group; C) TCE+ group; D) TCE- group; E) C3aRA/TCE+ group; F) C3aRA/TCE- group. Expression of C3aR was mainly located in glomerulus in TCE+ group. C3aRA pretreatment blocked the expression C3aR both in C3aRA/TCE+ group and C3aRA/TCE- group. G) Expression of C3aR mRNA in kidney was analyzed by qRT-PCR in different groups. Note: Compared to vehicle group, ^a $P < 0.05$; Compared to relevant TCE- group, ^b $P < 0.05$. There is no interaction between TCE and C3aRA in C3aR mRNA. The ANVOA table is shown in supplement data.

Fig. 4. C3aRA pretreatment alleviated over-expressed inflammatory cytokines in impaired kidney (400×).

A) Expression of IL-1 β , IL-6, and IL-23 in TCE+ group and C3aRA/TCE+ group via DAB staining; B) Average gray value of IL-1 β , IL-6, and IL-23 calculated by Image J in above groups. Note: Compared to vehicle group, ^a $P < 0.05$; Compared to TCE- group, ^b $P < 0.05$; Compared to C3aRA/TCE+ group, ^c $P < 0.05$. The principal simple effect is TCE treatment. There is interaction between TCE and C3aRA in IL-1 β , IL-6, and IL-23, by Two-way ANOVA. The ANVOA table is shown in supplement data.

Fig. 5. C3aR signaling regulates the accumulation of Th17 phenotype in local

impaired kidney.

A) Fluorescent double labeling of CD4 (red) and IL-17A (green) in the impaired kidney of TCE+ group and C3aRA/TCE+ group showed that massive overlap of CD4 and IL-17A was found in the sections of local impaired kidneys in TCE+ group, but not C3aRA/TCE+ group. B) Quantification of CD4 and IL-17A calculated by Image J in above groups. Note: Compared to vehicle group, ^a $P < 0.05$; Compared to TCE- group, ^b $P < 0.05$; Compared to C3aRA/TCE+ group, ^c $P < 0.05$. The principal simple effect is TCE treatment. There is interaction between TCE and C3aRA in quantification of IL-17A. The ANVOA table is shown in supplement data.

Fig. 6. C3aRA pretreatment suppressed STAT3 pathway in impaired kidney tissue.

A) Total STAT3 (t-STAT3), phosphorylated STAT3 (p-STAT3), and GAPDH proteins expressed in blank control group, vehicle group, TCE+ group, TCE- group, C3aRA/TCE+ group, and C3aRA/TCE- group; B) Relative protein level of t-STAT3 and C, Relative protein levels of p-STAT3 were calculated by Image J in above groups. Note: Compared to vehicle group, ^a $P < 0.05$; Compared to TCE- group, ^b $P < 0.05$; Compared to C3aRA/TCE+ group, ^c $P < 0.05$. The principal simple effect is TCE treatment. There is interaction between TCE and C3aRA in quantification of STAT3 and p-STAT3. The ANVOA table is shown in supplement data.

Table 1 Details of mice skin allergy and kidney pathological injury in different groups (n=50).

Group	n	Skin allergy				Sensitization rate	Kidney pathological injury	
		Score=0	Score=1	Score=2	Score=3		n	Incidence
Blank control group	5	5	0	0	0	0	0	0
Vehicle control group	5	5	0	0	0	0	0	0
TCE treatment group	20	11	4	4	1	45.0%	-	35%
TCE positive sensitization sub-group (TCE+)	9	0	4	4	1	-	7	-
TCE negative sensitization sub-group (TCE-)	11	11	0	0	0	-	0	-
TCE with C3aRA pretreatment group	20	13	3	4	0	35.0%	-	25%
TCE sensitization positive with C3aRA pre-treatment sub-group (C3aRA/TCE+)	7	0	3	4	0	-	5	-
TCE sensitization negative with C3aRA pre-treatment sub-group (C3aRA/TCE-)	13	13	0	0	0	-	0	-

Figure 1
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Figure 2
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A

B

C

D

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Supplement data

Table 1. AVOVA table depicting impact of “TCE treatment” and “C3aRA pre-treatment” on
different variables

Source	Mean Square	F value	P value
Variable: BUN			
TCE treatment	111.87	29.79	0.00
C3aRA pre-treatment	9.40	2.50	0.13
TCE treatment* C3aRA pre-treatment	41.52	11.06	0.00
Variable: CRE			
TCE treatment	8503.02	12.94	0.00
C3aRA pre-treatment	3637.86	5.54	0.03
TCE treatment* C3aRA pre-treatment	657.13	2.65	0.12
Variable: Serum IL-6			
TCE treatment	3920.00	420.38	0.00
C3aRA pre-treatment	369.80	39.66	0.00
TCE treatment* C3aRA pre-treatment	369.80	39.66	0.00
Variable: C3aR mRNA			
TCE treatment	3.34	88.54	0.00
C3aRA pre-treatment	0.01	0.24	0.63
TCE treatment* C3aRA pre-treatment	0.00	0.10	0.76
Variable: IL-1β			
TCE treatment	1429.79	194.22	0.00
C3aRA pre-treatment	345.12	46.88	0.00
TCE treatment* C3aRA pre-treatment	370.12	50.28	0.00
Variable: IL-6			
TCE treatment	6826.57	1265.00	0.00
C3aRA pre-treatment	623.20	115.47	0.00
TCE treatment* C3aRA pre-treatment	619.20	114.73	0.00
Variable: IL-23			
TCE treatment	3841.88	341.93	0.00
C3aRA pre-treatment	662.49	58.96	0.00
TCE treatment* C3aRA pre-treatment	620.90	55.26	0.00
Variable: CD4			
TCE treatment	3986.79	106.52	0.00
C3aRA pre-treatment	174.93	4.67	0.39
TCE treatment* C3aRA pre-treatment	5.19	0.14	0.71

Variable: IL-17A

TCE treatment	1225.00	66.62	0.00
C3aRA pre-treatment	802.78	43.66	0.00
TCE treatment* C3aRA pre-treatment	625.00	33.99	0.00

Variable: STAT3

TCE treatment	0.02	2.06	0.17
C3aRA pre-treatment	0.00	0.31	0.59
TCE treatment* C3aRA pre-treatment	0.19	25.51	0.00

Variable: p-STAT3

TCE treatment	93.36	124.87	0.00
C3aRA pre-treatment	22.57	30.18	0.00
TCE treatment* C3aRA pre-treatment	30.07	40.22	0.00

sFig. 1. Inflammatory mediators expressed in the local impaired kidney in the control and TCE negative sensitized mice. Scale bar indicated 50 μ m.

sFig. 2. Accumulation of Th17 phenotype in the local kidney in the control and TCE negative sensitized mice.

sFig. 3. A single dose of 10mg/kg SB290157 via intraperitoneal injection does not cause kidney damage in mice. Scale bar indicated 50 μm.