Title: Viral mimic polyinosine-polycytidylic acid potentiates liver injury in trichloroethylene-sensitized mice – viral-chemical interaction as a novel mechanism

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Abstract:

Occupational trichloroethylene (TCE) exposure can induce hypersensitivity dermatitis and severe liver injury. Recently, several clinical investigations indicate that viral infection, such as human herpesvirus-6, is associated with hepatic dysfunction in patients with TCE-related generalized skin disorders. However, whether viral infection aggravates TCE-induced liver injury remains unknown. This study aimed to explore the contribution of viral infection to the development of TCE-sensitization-induced liver injury in BALB/c mice. Female BALB/c mice were randomly assigned into four groups: solvent control group (n = 20), TCE group (n = 80), poly(I:C) group (n = 20) and combination of TCE and poly(I:C) injection (poly(I:C)+TCE) group (n = 80). Poly(I:C) (50μg) was i.p. administrated. TCE and poly(I:C)+TCE groups were further divided into sensitization and non-sensitization subgroup. Complement 3 and 3a protein levels, and complement factors were measured. Combination treatment significantly aggravated TCE-induced liver injury, decreased complement 3, but increased complement 3a in serum and liver tissues in sensitization group. These changes were not correlated with the hepatic complement 3 transcription. Moreover, combination treatment specifically promoted complement factor B, but not factor D and factor H expressions, in liver tissues. These data provide first evidence that Poly(I:C) potentiates liver injury in BALB/c mouse model of TCE-sensitization. Upregulated complement 3a and factor B contributes to the poly(I:C) action in TCE-induced liver injury. This new mode of action may explain increased risk of chemical-sensitization induced tissue damage by viral infection.

Key words: trichloroethylene, liver injury, polyinosine-polycytidylic acid, complement,
factor B
1. Introduction

Trichloroethylene (TCE), a common industrial solvent, is used in large quantity in some developing countries. Increasing evidence from epidemiology and laboratory animal studies indicates that TCE is an immunotoxicant and capable of disrupting human immune homeostasis, which results in autoimmune disease or even severe hypersensitive skin disorder (Chiu et al., 2013). It is well known that TCE-induced hypersensitivity is clinically characterized by serious generalized hypersensitivity dermatitis, often accompanied by lethal liver dysfunction, occurring among workers after a period of occupational TCE exposure (Kamijima et al., 2007; Xu et al., 2009). The prevalence of this illness is estimated to be as high as 13% and the morality is about 9-13% (Kamijima et al., 2007). For effective prevention and clinical management, this life-threatening syndrome has been classified as occupational disease and is termed as Occupational Dermatitis Medicamentosa-like of Trichloroethylene (ODMLT) (Kamijima et al., 2007).

Apart from the distinct cutaneous symptoms, epidemiological investigations show that the symptoms of infection such as fever and headache are frequently present in ODMLT patients at early stage (Xu et al., 2009). These clinical manifestations suggest that ODMLT may begin with infection. Interestingly, several studies have proposed that herpesvirus and cytomegalovirus are reactivated in a considerable proportion of the ODMLT cases with liver damage. Furthermore, the antibody titer against virus was significantly higher in the patients (Kamijima et al., 2013; Watanabe et al., 2010).

Pathogenic invaders can act as danger signals by engaging pattern recognition
receptors (PRRs) and activating innate immune system, which results in the expression of pro-inflammatory cytokines (Bowie and Unterholzner, 2008). There is also evidence that adapted immune response, participates in the pathology of ODMLT, which can be activated and amplified by priming T cells and inducing antigen specific responses (Bowie and Unterholzner, 2008). Therefore it is possible that the original infection caused by virus may be involved in the initiation of the disease.

Many studies suggest that complement system plays an important role in ODMLT (Huang et al., 2014; Yue et al., 2007; Zhao et al., 2012). The complement cascade can be activated by classical, alternative and lectin pathways and all these routes lead to the cleavage of the central molecule, complement component 3 (C3), to C3a and C3b (Kolev et al., 2014). The C3 is mainly synthesized by liver, although it has been suggested that almost all cells in the body have the capacity to generate complement proteins in human (Kolev et al., 2014). It is known that the level of C3 dramatically decreases in serum during the early outset of disease and recovers to the normal level after a period of stay in the hospital following the admission among ODMLT patients (Huang et al., 2014). Moreover, the decrease of C3 was significantly associated with the extent of liver injury induced by TCE (Huang et al., 2014). Considering the biochemical process of C3 activation, these C3 changes may be attributed to the balance between C3 cleavage and production. There is report that increased breakdown of C3 can be detected in damaged liver tissue from TCE-sensitized mice with higher depositions of downstream complement fragments and the ultimate membrane attack complex (Wang et al., 2014; Zhang et al., 2013). In
addition, elevated levels of C3a-desArg in circulation are also observed with liver impairment in TCE-sensitized mice (Zhang et al., 2013). Furthermore, there is no clear evidence to suggest that hepatic C3 generation is suppressed among ODMLT cases. Therefore, we propose that excessive complement activation occurring during the progress of ODMLT, which may consume the circulated C3 protein and lead the declined serum C3 level, contribute to concurrent liver injury.

In our previous studies, we have established a TCE sensitization model using BALB/c mouse, which will be used to explore the mechanism of liver injury induced by TCE (Wang et al., 2014; Wang et al., 2015). This TCE sensitization-induced liver injury only occurs in TCE sensitization-positive group and is characterized by hepatocyte swelling, deposition of cytokines, activation of complement system, mild increments of ALT and AST, and recovery when TCE exposure is ceased (Wang et al., 2014; Zhang et al., 2013). This pathological feature is different from classic liver injury commonly induced by hepatotoxicants via direct chemical toxicity. In the present study, we hypothesize that the initial viral infection aggravates liver injury in TCE-sensitized BALB/c mice. Polyinosine-polycytidylic acid (polyI:C) is structurally similar to double-stranded RNA generated by virus and is widely used as a virus mimic in experimental studies (Cheng et al., 2009). Therefore, we employ polyI:C as a virus mimic to investigate whether it could exacerbate liver injury and activate C3 in TCE-sensitized BALB/c mice.

2. Material and Methods
2.1 Treatments of Animals

Female BALB/c mice (6-8 weeks) were purchased from the Experimental Animal Center of Anhui Province (Anhui, China) and maintained with a 12 h light/dark cycle in a controlled temperature (20–25 °C) and humidity (50 ± 5%) environment with free access to food and water. Animals were given one-week adaptation before the treatment. The dorsal hair of the mice was shaved with an area about 4 cm² and remained bald throughout the experiment by shaving regularly. All experiments were performed in accordance with guidelines from Animal Care and Use Committee of Anhui Medical University.

Workers are commonly exposed to TCE through direct skin contact. This route of exposure was mimicked by cutaneous application of TCE on mice. In TCE treatment group, chemical sensitization was induced according to our previous protocol (Wang et al., 2014; Wang et al., 2015; Zhang et al., 2013). Briefly, 80 mice were randomly assigned into four subgroups (n = 20 for each subgroup) of different time points (day 20, 21, 22 and 26), and received the same treatment protocol as follows. On the first day, mice were intradermally injected with 100μl mixture of 50% TCE and Freund complete adjuvant (Sigma Aldrich, St. Louis, MO, USA) with the volume ratio of 1:1 within the area of the naked back skin. The 50% TCE was prepared by mixing TCE, acetone and olive oil (all from Sigma Aldrich, St. Louis, MO, USA) with a volume ratio of 5:3:2. On day 4, 7 and 10, 100μl 50% TCE was painted on the same area and the area was then covered with a filter paper, secured by a non-irritating tape. On day
17 and 19, 30% TCE (TCE: acetone: olive oil= 3:5:2) was applied as the first and second challenge, respectively.

Viral infection is closely associated with TCE-induced hypersensitivity syndrome(Kamijima et al., 2013; Watanabe et al., 2010; Xu et al., 2009), and this infection was mimicked by poly(I:C) injection, which is widely used as a simulation of viral infection in animal model(Cheng et al., 2009). In poly(I:C) and TCE cotreatment group, another 80 mice were randomly assigned into four subgroups of different time points (n = 20 for each subgroup) followed by the same treatment. 50μg poly(I:C) (Invivogen, San Diego, California, USA) was dissolved in 0.2ml sterile saline and then an intraperitoneal injection was given three hours before the second challenge (FIG 1). In order to ensure comparability, mice in TCE-treated group were also administrated with the same sterile saline as in other groups before the second challenge. In poly(I:C) control group, poly(I:C) and all the above solvents and saline were used. Solvent control group received only solvent and saline injections.

On day 20, 21, 22 and 26, animals were euthanized according to the subgroup assigned. The cutaneous response was scored before euthanasia according to a four-point scale: 0 (no reaction), 1 (scattered mild redness), 2 (moderate and diffuse redness) and 3 (intensive erythema and swelling). If the score number was no less than one, the corresponding mouse was categorized as sensitized. Otherwise, it would be identified as non-sensitized.

2.2 Evaluation of liver injury
For biochemical analysis, the blood sample was collected by retro-orbital puncture and placed under room temperature for two hours for clotting. The serum was separated by centrifugation at 10,000g for 15min and the supernatants were collected. The level of alanine aminotransferase (ALT) was determined by an automated biochemical analyzer. For histological pathology evaluation, tissue from the right lobe of the liver was taken and fixed in 4% neutral buffered formaldehyde overnight. The liver samples were then embedded in paraffin. The liver tissue block was sliced at 5μm thickness, dewaxed and stained with hematoxylin and eosin for histological examination. The remaining fresh liver tissue was cryopreserved for subsequent experiments.

2.3 ELISA assay

Serum levels of C3, C3a, factor B, factor D and factor H were measured using respective commercial ELISA assay kits, and following manufacturer instructions (Elabscience, Wuhan, China). The optical density (OD) of each sample well was read at 450 nm using a microplate reader (BioTek, Winooski, VT, USA).

2.4 Immunohistochemical detection and scoring

For immunohistochemical assay, sliced tissue was dewaxed and rehydrated using the routine protocol. After antigen retrieval and endogenous peroxidase block, tissues were incubated with C3 antibody (MP Biomedicals Headquarters, Santa Ana, CA), C5b-9 antibody (Abcam, Cambridge, MA, USA), factor B antibody (Abcam,
Cambridge, MA, USA) overnight at 4°C. Thereafter, slides were incubated with horseradish peroxidase-labeled avidin-biotin complex (Dako, Carpinteria, CA, USA). The immuno-binding products were visualized by diaminobenzidine and counterstained by hematoxylin. To make a quantitative evaluation, two investigators independently took photos of 10 random visual fields under ×400 magnifications from each sample by microscopy (Olympus, Center Valley, PA, USA).

Immunohistochemical staining was scored based on Histoscore (H-score). This involved a semiquantitative assessment of both the intensity of staining (graded as: 0, non-staining; 1, weak; 2, median; or 3, strong) and the percentage of positive cells. The values of scores ranged from 0 to 300.

2.5 Total RNA Isolation and real-time RT-PCR

Total RNA was extracted by using Trizol Reagent (Invitrogen, Grand Island, NY, USA) from liver homogenates. The cDNA was yielded with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Grand Island, NY, USA). Real-time qualitative PCR was performed using LightCycler 480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) and the respective primers. Primer sequences are as follows: gapdh F’-ACC CCA GCA AGG ACA CTG AGC AAG, R’-GGC CCC TCC TGT TAT TAT GGG GGT; c3 F’-CAA CGC AAG TTC ATC AGC CA, R’-CAA CGC AAG TTC ATC AGC CA. The amplification reactions were conducted on LightCycler® 480 Instrument (Roche Diagnostics GmbH, Mannheim,
Germany). The PCR parameters were: 95 °C for 5 min, 40 cycles PCR (95 for 15 s, 60 °C for 15 s, and 72 °C for 30 s).

2.6 Statistical analysis

All data were presented as mean ± standard deviation (SD). Two-tailed Student $t$ test was used to compare two independent groups. Comparisons among multiple groups were performed by ANOVA with a post hoc test of significance between individual groups. P value less than 0.05 was considered to be statistically significant.

3. Results

3.1 PolyI:C potentiated the liver injury in TCE-sensitized BALB/c mice

PolyI:C is structurally similar to double-stranded RNA generated by virus. Previous study indicates that i.p. injection of 50μg polyI:C alone into BALB/c mice does not cause apparent liver damage (Cheng et al., 2009). Therefore, we used this dose in the present study. As expected, administration of 50μg polyI:C alone (4 mice each day, n = 16) did not affect liver function (ALT: 15.30±3.76; 17.74±2.29; 14.34±3.58; 18.66±1.22, at day 20, 21, 22 and 26 respectively:), which was comparable to solvent control group (4 mice for each day, n = 16; ALT: 19.56±3.24; 18.54±1.86; 18.49±5.37; 17.42±2.31, at day 20, 21, 22 and 26 respectively). The normal histological micrographs in two control groups are shown in the supplement figure. TCE alone treatment resulted in about 33.3% sensitization rate and moderate liver impairment in the sensitized mice. The levels of ALT were mildly elevated in the TCE-sensitized
group, and peaked at 72 hours after the last challenge (FIG 2A). Histological pathology evaluation indicated that the swelling hepatocytes and neutrophil infiltration were occasionally identified in this tissue (FIG 2B). However, polyI:C and TCE cotreatment resulted in the sensitization rate of TCE reach to about 40% in BALB/c mice, although there was no statistical significance in comparison to TCE alone treatment group. In addition, the biochemical measurement indicated that the level of ALT in the sensitized mice from the polyI:C and TCE cotreatment group was significantly increased, when compared with that in the sensitized mice from the TCE alone treatment group, and peaked earlier at 48 hours (FIG 2A). In line with that, histological pathology evaluation also showed that there was severe and massive swelling and ballooning degeneration of hepatocytes with inflammatory cells infiltration in liver tissue(FIG 2B). Of note, these changes only occurred in sensitization positive groups and therefore suggested that the action was sensitization-specific.

3.2 PolyI:C potentiated the decrease of serum complement 3 in TCE-sensitized BALB/c mice

Several population investigations show that the level of serum C3 was dramatically decreased among ODMLT patients(Huang et al., 2014; Yue et al., 2007; Zhao et al., 2012). Although serum C3 cannot be considered as truly hepatic, this protein is predominantly synthesized by liver in mouse and human (Fagerberg et al., 2014; Huang et al., 2014). Therefore, we tested the possibility that C3 decrease could have
been attributed to the severe liver injury, which impaired hepatic C3 generation. Our data also showed that the content of serum C3 significantly was reduced in sensitized mice of polyI:C and TCE cotreatment group, when compared with that in the sensitized mice from the TCE alone treatment group. The most significant reduction of C3 was observed at 48 hours. The effect only occurred in the sensitization-positive cotreatment group hence suggesting a sensitization-specific effect. Of note, TCE alone treatment was insufficient to reduce the content of C3 in serum (FIG 3A). In order to answer whether the ability of C3 production of the liver was damaged, we detected the expression of C3 at the transcriptional levels. Our results showed that there was no significant difference between any groups at each selected time point (FIG 3B). These data together suggest that C3 may play a role in PolyI:C potentiated the liver injury in TCE-sensitized BALB/c mice, and its decrease occurred at the post-transcription.

3.3 PolyI:C enhanced TCE-induced complement 3a and factor B increases in serum

A further possibility was that, excessive C3 cleavage could cause massive consumption of C3, thereby inducing apparent serum C3 reduction(Kolev et al., 2014). Therefore, we detected the level of C3 cleavage product, C3a, in serum at different times. The results showed that the level of C3a indeed dramatically increased in serum, which reached to the highest level at the same timer point i.e., at 48 hours, in sensitized mice from polyI:C and TCE cotreatment group. However, the level of C3a
only slightly elevated in TCE-sensitized group with a plateau from 48 to 72 hours (FIG 3C).

Since factor B, factor D and factor H are considered to participate in the regulation of complement activation. We therefore determined the levels of factor B, factor D and factor H in the serum. As shown in FIG 4, the expression of factor B was up-regulated on day 1 and 2 by the cotreatment, whereas factor D and factor H showed no significant difference between each group at each selected time point.

The above effects were again not observed in the sensitization-negative group under the cotreatment, therefore suggesting a sensitization-specific effect.

### 3.4 Complement activation contributed to PolyI:C potentiation of TCE-induced liver injury

Our previous studies indicated that local complement activation occurred in the mild liver injury induced by TCE sensitization(Wang et al., 2014; Zhang et al., 2013). To investigate whether the local complement activation also participated in PolyI:C potentiated, TCE-induced liver injury, we examined the depositions of C3 fragments and membrane attack complex (MAC) in the liver tissue. No apparent depositions of C3 fragments and membrane attack complex were observed in solvent and polyI:C alone treatment groups (see Supplement data). As shown in Figure 5 and 6, there were markedly increased depositions of C3 fragments and membrane attack complex in sensitized mice from polyI:C and TCE cotreatment group, in comparison with the
sensitized mice from TCE alone treatment group, suggesting complement was strongly activated in polyI:C and TCE cotreatment group. The effect was not seen with the co-treatment but sensitization-negative group.

3.5 Hepatic complement factor B increase by PolyI:C potentiation.

The increased expression of complement factor B was reported to promote the regional and systematic complement activation(Kaczorowski et al., 2012; Ostvik et al., 2014; Schnabolk et al., 2015; Zou et al., 2013). Therefore, we hypothesized that the hepatic complement factor B generation was up-regulated in the polyI:C and TCE cotreatment group, which triggered excessive complement activation. As our results showed, no apparent factor B expression was found in solvent control and polyI:C group (See supplement data). The expression of factor B in the liver was however significantly increased in the sensitized mice from polyI:C and TCE cotreatment group, especially at 48 hours. This effect was again sensitization-specific as it was not seen with the cotreatment but sensitization-negative group. There were no apparent depositions of factor B in TCE only treatment group (FIG 7), emphasizing an interaction between poly I:C and TCE.

4. Discussion

Occupational TCE exposure may lead to generalized hypersensitive dermatitis and life-threatening complications such as serious liver injury (Kamijima et al., 2007; Xu et al., 2009). Clinical observations show that the symptoms of infection such as fever
and headache often occur at the early stage, which indicate that a preceding infection may contribute to the progress of this disease (Xu et al., 2009). In the present study, we pretreated the mice by i.p. injection of poly(I:C), a commonly used virus mimic due to the feature that it is structurally similar to viral double-stranded RNA, to mimic the systematic viral infection that would attack patients at early stage (Matsumoto and Seya, 2008). Our results firstly show that poly(I:C) potentiates the liver damage in mice following sensitization by TCE without affecting the sensitization rate, via a mechanism of activation of complement C3a and factor B. These findings demonstrate that virus infection contributes to TCE-induced liver damage through specific complement-dependent mechanisms. To our knowledge this is the first study to show that viral infection can precipitate the liver damage induced by chemical sensitization and this have important implications in understanding how environmental chemicals affect human body.

It has been proposed that complement system plays an important role in TCE sensitization and the accompanied liver damage. Clinical evidence indicates that the serum level of complement 3 in ODMLT patients decreases dramatically during the early outset of the disease but recovers to normal level sometime after hospital admission (Huang et al., 2014). Moreover, the magnitude of C3 reduction is significantly associated with the severity of the liver injury (Huang et al., 2014). In the present study, our results showed that TCE treatment alone is inadequate to reduce the level of serum C3 content, while poly(I:C) and TCE cotreatment significantly reduced serum C3, suggesting an interaction between poly (I:C) and TCE.
Furthermore, we tested the possibility that clinical serum C3 suppression could have been attributed to the impaired C3 generation induced by severe liver injury.

Interestingly, the transcriptional levels of C3 in liver tissue from each group were comparable, suggesting that aberrant C3 production was unlikely to have happened and participated in this phenomenon. Therefore, it is reasonable to postulate that the cotreatment-induced sensitization enhances complement activation, thus consuming more C3 molecule and resulting in the systemic C3 reduction. This would also predict that the activated form of C3, i.e., C3a would increase. As expected, significantly increased C3 fragments and MAC molecule were identified in liver tissue in the TCE-sensitized group. More importantly more intense and widespread depositions of these two proteins were detected in the cotreatment and sensitization-positive group. In addition, the levels of circulating C3a were also consistently elevated. Hence, excessive local complement activation may contribute to the circulating C3 reduction and the liver damage by TCE and its potentiation by poly (I:C).

Previous studies document that up-regulation of complement factor B, factor D and factor H in both immune cells and parenchyma cells causes increased complement activation(Kaczorowski et al., 2010; Ostvik et al., 2014; Zou et al., 2013). In this study we subsequently investigated whether the generation of factor B, factor D and factor H was involved in this study. Our results showed that the expression of factor B, but not factor D and factor H, in the liver was significantly increased in the sensitized mice of poly(I:C) and TCE cotreatment group, while the its levels were comparable between TCE-sensitized group and control groups. This suggests that the different
levels of complement activation may be dependent on distinct mechanisms between
the TCE-sensitized group and the cotreatment-sensitized group. The latter may be
partly attributed to the up-regulation of factor B. TLRs-related pathways may be
involved in this process. Studies show that inhibition of NF-κB and JNK signaling can
completely or partially block the stimulation of factor B induced by poly(I:C)
treatment in vitro (Kaczorowski et al., 2010). Trif knock-out mice also have reduced
capacity to generate more factor B upon TLR4 activation (Zou et al., 2013). Moreover,
MyD88 deletion entirely abolishes this ability caused by TLR2 stimulation (Zou et al.,
2013). However, in the present study, poly(I:C) alone application, which has been
reported to initiate TLR3 signaling and subsequent factor B rise immune cells and
parenchyma cells from several in vitro experiments (Kaczorowski et al., 2010; Ostvik et
al., 2014; Zou et al., 2013) and one in vivo experiment (Kaczorowski et al., 2010), did
not cause factor B enhancement. This suggests that this effect is tissue-specific and
may also reflect the difference between in vitro and in vivo conditions. Time of
observation is another factor. Changes to factor B expression in immune and
parenchyma cells were most apparent at 6-24 hours after TLR3 ligation in vitro
(Kaczorowski et al., 2010; Ostvik et al., 2014; Zou et al., 2013) and peaked at 6 hours
after poly (I:C) stimulation in vivo (Kaczorowski et al., 2010); therefore the level of
this protein may have recovered after 24-48 hours under in vivo treatment as seen in
our experiment. TCE treatment alone in our experiment did not cause a significant
rise in factor B. However, the co-treatment caused an increase in factor B. This clearly
shows that there is an interaction between poly (I:C) and TCE. Whilst Poly (I:C) may
have initially stimulated factor B increase to a sub-threshold level for detection, TCE could have enhanced and sustained the increase. Indeed, activation of MAPK and NF-κB pathways, which are crucial in promoting factor B synthesis (Kaczorowski et al., 2010), is identified in TCE-sensitized mice in our previous study (Zhang et al., 2015). Thus, TCE sensitization may provide a favorable environment for sustained production of B stimulated by Poly (I:C) through MAPK and NF-κB pathways.

It is interesting to note that all the co-treatment related changes in the present study occurred only in the co-treatment and sensitization positive group but not in the co-treatment whilst sensitization-negative group. This demonstrates that the effects by poly(I:C) and TEC interaction are sensitization-specific hence the immune responses.

There is limited study on viral and chemical interactions in environmental chemical induced sensitization. There are observations that certain viral infection may aggravate asthma although the mechanisms are poorly understood (Kloepfer and Gern, 2010). Our findings suggest that viral-chemical interaction may also apply to some other environmental chemicals. A better understanding of virus-aggravated sensitization by exposure to harmful environmental chemicals may lead to more effective public health strategy to limit chemical damage to the general population.

In summary, poly(I:C) significantly potentiates the liver injury induced by TCE sensitization and decreases the level of serum complement C3, which is consistent with the clinical observations among ODMLT patients. Although poly(I:C) does not impair the ability of liver to generate C3, it causes massive complement activation and
its deposition in the liver tissue. Increased complement C3a and up-regulation of factor B are the underlying mechanisms for poly(I:C) potentiation of TCE sensitization-induced liver injury.
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References:

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**Figure legends:**

Figure 1. Flow diagram of the treatment procedure. Initially, the hair on the back of female BALB/c mice was removed by shaving and then treated with intradermal injection of the mixture that consisted of 50μl 50% TCE (TCE: acetone: olive oil= 5: 3: 2) and 50μl Freund complete adjuvant on day one. Then mice were sensitized by applying 100μl 50% TCE on the naked skin on day 4, 7 and 10. Subsequently the challenge phase was carried out by applying 100μl 30% TCE (TCE: acetone: olive oil= 3: 5: 2) on the naked skin on day 17 and 19. During this stage, 50μg poly(I:C) was i.p. injected 3 hours prior to the second administration at day 19. Finally, mice were classified into sensitized and non-sensitized ones according to the score of cutaneous response, followed by euthanasia and sacrifice on day 20, 21, 22 and 26.

Figure 2. PolyI:C potentiated the liver injury in TCE-sensitized BALB/c mice. A) Serum ALT levels in different treatment groups; data showing TCE sensitization induced elevation of ALT and its augmentation by Poly I:C. B) Histopathological changes in the liver; representative microscopic pictures (magnification, 400×) of the HE staining showing widespread ballooning degeneration in the sensitized mice of the cotreatment group. Scale bar represented 50 μm. a $P<0.05$ compared with solvent group; b $P<0.05$ compared with sensitized mice in TCE group. Note: No histological changes were found in solvent and poly(I:C) and
groups and, in order to represent results more concisely and clearly, data and pictures of the two groups were not shown.

Figure 3. Decreased circulation C3 and increased C3a in the sensitized mice of poly(I:C) and TCE cotreatment group. A) Serum levels of C3 (mean ± SD) in each group. B) Transcription of C3 in liver tissue by real-time RT-PCR. C) The level of C3a in the serum. a $P<0.05$ compared with solvent group; b $P<0.05$ compared with sensitized mice in TCE group. In order to represent the results more concise and clear, data from solvent and poly(I:C) groups were not shown.

Figure 4. The expression of factor B, factor D and factor H in the serum by ELISA assay. The serum levels of factor B increased significantly in sensitized mice from cotreatment group at 24 and 48 hour. a $P<0.05$ compared with solvent group; b $P<0.05$ compared with sensitized mice in TCE group. In order to represent the results more concise and clear, data from solvent and poly(I:C) groups were not shown.

Figure 5. The expression of C3 fragments in the liver by immunohistochemistry. A) Sections from right liver lobe were incubated with anti-C3 antibody and this protein was visualized with diaminobenzidine. The representative microscopic pictures (magnification, 400×) were given from each group. Scale bar represented 50 μm. B) Immunohistochemical score of each group on each day. Bar represented the median score of a group. Note: No histological changes were found in solvent and poly(I:C) groups and data are shown in supplement file.
Figure 6. The expression of C5b-9 in liver tissue by immunohistochemistry. A) Liver tissue sections were incubated with anti-C5b-9 antibody and the binding was visualized with diaminobenzidine. The representative microscopic pictures (magnification, 400×) were presented from each group. Scale bar represented 50 μm. B) Immunohistochemical score of each group on each day. Bar represented the median score of a group. Note: No histological changes were found in solvent and poly(I:C) groups and data are shown in supplement file.

Figure 7. Immunohistochemical detection of the expression of factor B in the liver. A) Liver sections were incubated with anti-factor B antibody and the protein-antibody complex was visualized with diaminobenzidine. The representative microscopic pictures (magnification, 400×) were shown from each group. Scale bar represented 50 μm. B) Immunohistochemical score of each group on each day. Bar represented the median score of a group. Note: the solvent and poly(I:C) groups presented normal liver histology (data given in supplement file).
Figure 1

Sensitization phase: 50% TCE application each time

Elicitation phase: 30% TCE application each time

Classification of sensitization and sacrifice later (20 mice each day)

1 4 7 10 17 19 20 21 22 26 (time, day)

Intradermal injection of 50% TCE and FCA mixture (N = 80)

i.p. injection of 50 μg poly(I:C) 3 hours ahead
Figure 2

A

![Graph showing ALT (U/L) over time (d) for different groups.]

B

![Images showing liver tissue samples for different groups over days 1, 2, 3, and 7.]
Figure 4

(A) Bar graph showing Factor B (ng/ml) over days post elicitation (d) for different conditions:
- TCE(-)
- TCE(+)
- Poly(I:C)+TCE(-)
- Poly(I:C)+TCE(+)

(B) Bar graph showing Factor D (ng/ml) over days post elicitation (d) for different conditions:
- TCE(-)
- TCE(+)
- Poly(I:C)+TCE(-)
- Poly(I:C)+TCE(+)

(C) Bar graph showing Factor H (ng/ml) over days post elicitation (d) for different conditions:
- TCE(-)
- TCE(+)
- Poly(I:C)+TCE(-)
- Poly(I:C)+TCE(+)
Figure 5

A

<table>
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<th>day 1</th>
<th>day 2</th>
<th>day 3</th>
<th>day 7</th>
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<tr>
<td>TCE</td>
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<tr>
<td>Poly(I:C) + TCE</td>
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<td>sensitization group</td>
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<tr>
<td>TCE</td>
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<tr>
<td>Poly(I:C) + TCE</td>
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</tbody>
</table>

B

H-score of C3 fragments

- TCE(-)
- Poly(I:C)+TCE(-)
- TCE(+)
- Poly(I:C)+TCE(+)

days post elicitation (d)