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Immunological Profiles of Mice Protected from Chlamydia-induced Infertility by Anti-caspase Treatment

C. E. Ukwade[1], O. A. T. Ebuehi[1*], J. U Igietseme[2], S. Ouburg[2], J. A. Land[2], Y. Omosun[2], K. Joseph[2], J. Partin[2], Q. He[2], F. O. Eko[2], D. Ellerson[2], C. Bandea[2], S. A. Morre[2], G. Zhong[2] and C. M. Black[2]

[1]Department of Biochemistry, College of Medicine, University of Lagos, Lagos, Nigeria.
[2]Department of Microbiology, Biochemistry and Immunology, Morehouse School of Medicine, Atlanta, Georgia, USA.

Authors' contributions

This work was carried out in collaboration between all authors. Authors CEU, OATE and JUI designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors SO, JAL, YO, KJ, JP, QH, FOE and DE managed the literature searches and analyses of the study performed the spectroscopy analysis. Authors CEU, OATE, JUI, FOE, YO, CB, SAM, GZ and CMB managed the experimental process. All authors read and approved the final manuscript.

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ABSTRACT

The study is to investigate the effect of anti-caspase treatment on anti-chlamydia immune response in mice. Both the humoral and aspects of cell-mediated immune response against *Chlamydia trachomatis* were studied. Antibody response was measured using the ELISA technique to identify all common isotypes, and cytokine response was measured using the PCR technique. The antibody levels (IgG, IgG1, IgG2a and IgA) in Z-VAD-FMK treated group were significantly higher than non-treated group. ELISA results [showed a significantly higher amount of antibodies (IgG, IgG1, Ig G2a

**Corresponding author: E-mail: ebuehi@yahoo.com, oebuehi@unilag.edu.ng, oatebuehi@cmul.edu.ng;*

and IgA)] were produced in the mice that were pre-treated with Z-VAD-FMK before infection with *Chlamydia trachomatis* compared to mice post treated with Z-VAD-FMK after *Chlamydia trachomatis infection*. Data of the study indicate that the caspase inhibitor, Z-VAD-FMK did not negatively affect humoral and T cell mediated immune responses against *C. trachomatis* in mice.

Keywords: Chlamydia trachomatis; anti-caspase treatment; immune response; infertility; mice.

1. INTRODUCTION

The obligate intracellular bacterium *Chlamydia trachomatis,* continue to pose a considerable public health challenge worldwide. The major diseases are tubal factor infertility, ectopic pregnancy, pelvic inflammatory disease, conjunctivitis, blinding trachoma, non-gonococcal urethritis, cervicitis and interstitial pneumonia [1,2]. Chlamydiaeare major human pathogens. Their unique and complex reproductive cycle can enable the effective evasion of the host's defense mechanisms, leading to persisting infection. Immune responses stimulated by *Chlamydial* infection can result in tissue damage and scar formation, particularly upon re-infection [3-6].

The epidemic of *C. trachomatis* genital tract infections has not been controlled despite medical interventions including screening and treatment, but it is clear that the host immune response of humans and animals is capable of clearing infection, or at least controlling it asymptomatically at a level below that detectable with current diagnostic assays. Chlamydial genital infection is a common sexually transmitted disease that mostly goes undiagnosed because of its frequent asymptomatic presentation. Therefore unrecognized and untreated, the insidious infection promotes an acute or chronic inflammation causing tissue damage, pelvic inflammatory disease (PID), ectopic pregnancy, tubal factor infertility [7,8]. The latest international estimates of the genital infections worldwide show that approximately 92 million new cases of Chlamydial infections occur every year [1].

Studies on the natural course of untreated *C. trachomatis* lower genital tract infections in women show spontaneous clearance rates of 30% in the first weeks to months, 50% in 1 year, 80% in 2 years, and 94% in 4 years [4]. Although this is often the case, Chlamydial infection induces an intense and chronic inflammation, which may result in the tubal damage during this period. The clearance of the microorganisms depends on both a normal immune response and an antibiotic treatment. However, some women are not able to clear the pathogen adequately and become asymptomatic. Repeated infections can be even more damaging for women, because they accelerate the onset of serious sequelae in the reproductive tract, which may lead to pelvic inflammatory disease (PID) and infertility. The pathogenesis of Chlamydial disease in the female reproductive system involves the deleterious effects of host immune response against the infection [9-11].

The immune-pathogenesis of Chlamydial disease has led to the current hypothesis that the immune response to *C. trachomatis* in infected women is involved in both immunity and pathology [1,8]. The host response is not always able to control and resolve *C. trachomatis* infection. In fact, in some individuals, if the microorganism is not adequately treated, it remains for long periods in the infected subjects, leading to complications such as scaring, PID and tubal factor infertility [4]. The pathogenesis of infertility induced by Chlamydia infection is not well known, but studies implicate host inflammatory responses to bacteria.

Igietseme [9] previously investigated the hypothesis that tubal caspase activation during genital infection in females causes the distruption of key promoters of reproductive fertility which are required for embryo development and implantation. Key local factors critical for reproductive fertility, such as Dicer and the intestinal cells of Cajals, are either targets for caspase cleavage or destroyed through caspase-mediated apoptosis [3]. It was found that caspase-induction is required for Chlamydial induced infertility and local caspase inhibition could prevent infertility [2,9].

The objectives of this study are to analyze the immunological profiles of mice protected from Chlamydia-induced infertility by anti-caspase treatment by determining: the humoral and T cell-mediated immune responses against *C. trachomatis* after genital infection, as well as the

effect of caspase inhibitor on antibody and cytokine responses against *C. trachomatis.*

2. MATERIALS AND METHODS

2.1 Animals

Forty C57BL/6J mice 5-8 weeks old were obtained from The Jackson laboratory (Bar harbor MA) were used in this study, and 5 C57BL/6Jmale mice were used at a ratio 1 male to 2 female from the mating part of the experiment. The mice were fed on standard feed, and weighed 20±2.0 g, and when pregnant 30±2.0 g. The mice were divided into four groups; non-infected, infected-infertile pre-treatment, post-treatment with anti-caspase inhibitor (Z-VAD-FMK). All mice had mating and non-mating groups. The guidelines for ethical conduct in the care and use of animal research were strictly adhered to in accordance with the APA [12].

2.2 Administration

C. trachomatis stocks, animal infection and assessment of infertility. Stocks of *C. trachomatis* serovar L2 were propagated in Hela cells. The purified elementary bodies (EBs) were tittered as inclusion-forming units per milliliter (IFU/ml) by standard procedure. Female mice, infected intravaginally with 1*(10)5 IFU per mouse with the Chlamydia strain. The course of the infection was monitored by periodic cervico-viginal swabbing of mice and tissue culture isolation and enumeration of inclusions by standard immunofluorescence. Treatment with the pan-caspase inhibitor (Z-VAD-FMK) was performed before and after infection by administering 0.02 ML in PBS intravaginally under anesthesia. Chlamydia-induced infertility was assessed by mating infected mice and scoring for the number of pregnancies and the average number of pups in the different groups (Fig. 1). Mice were sacrificed by carbon dioxide asphyxiation cervical dislocation, blood was collected after the mice were sacrificed from the heart. The oviduct was collected and stored at -800C.The serum was collected after centrifugation at 2000 rpm for 10 min at 4℃. At the end of the mating, the following immunological analyses were performed: analysis of sera for anti-Chlamydial antibodies (all isotypes) by Enzyme-Linked Immunosorbent Assay (ELISA) and analysis of tissue lysates for Th1/Th2 cytokines and other cytokine by Polymerase Chain Reaction (PCR).

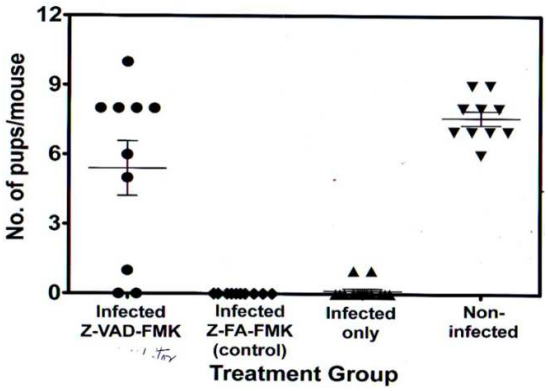

Fig. 1. Prevention of infertility in Chlamydial infected mice by local pan-caspase inhibitor. Infected and non-infected were mated to score for fertility

(Z-VAD-FMK: Pan-caspase inhibitor, Z-FA-FMK: Control)

2.3 Sample Preparation

Mice were divided into different groups as shown in Table 1. Naive (uninfected mice that were not treated with anti-caspase inhibitor) and positive control (mice that has been infected with *C. trachomatis* but not treated with anti-caspase inhibitor). The other mice were divided into two groups, pre and post treatment with caspase inhibitor (Z-VAD FMK). The post-treatment group was treated with caspase inhibitor (Z-VAD FMK) after infection with *C. trachomatis* accordingly: Post D+1 (day 1 after infection), Post D+7 (day 7 after infection), and Post D+14 (day 14 after infection). The pre-treatment group was treated with caspase inhibitor (Z-VAD FMK) before infection with *C. trachomatis* accordingly: Pre D-1 (day 1 before infection), Pre D-7 (day 7 before infection) Pre D-14 (day 14 before infection). The last set were the mating mice that were also divided into pre and post treatment with caspase inhibitor (Z-VAD FMK) and are represented in Table 1.

2.4 ELISA

The humoral immune responses against *C. trachomatis* were determined by measurement of antibody response, identifying different isotypes (IgG, IgG1, IgG2a and IgA), using the immunological (ELISA) techniques to analyze sera. Maxisorb plates were coated with Chlamydial Ag (10 ug/ml) in 0.10 ml PBS. PBS was added to control wells. Standard wells were

Table 1. The different groups of mice treated with caspase inhibitor

Entry#	Sample ID	Sample type
1	Post D+1 (Z-VAD- FMK)	
2	Post D+7 (Z-VAD- FMK)	
3	Post D+14 (Z-VAD- FMK)	
4	Pre D-1 (Z-VAD- FMK)	
5	Pre D-7 (Z-VAD- FMK)	
6	Pre D-14(Z-VAD- FMK)	Serum

coated with appropriate antibody isotype (IgG, IgG1, IgG2a and IgA) to cover 8 points, from 0ng/ml through 1000 ng/ml, and plates were left at 40C overnight. The plates were washed 3 times and 200 ul of Blocking Reagent was added into each well. This was incubated for 1 h 30 min at room temperature and plates were then washed twice. 0.05 ml of two-fold serially diluted samples was added to indicated wells and control and standard wells receive the same volume of PBS. This was incubated at room temperature for 2 hours. 0.0l ml of HRP-conjugated goat anti-mouse IgG, IgG1, IgG2a or IgA (as applicable) was added and this was incubated for 1 hour and then washed 3 times. 0.1 ml of HRP substrate (TMB) was added and incubated in the dark for 30 min. 0.05 ml of stop solution was added and absorbance was read at 492 nm. The immunological markers were then computed.

2.5 RT-PCR

Aspects of cell-mediated immune responses against *C. trachomatis* were determined by measurement of cytokines response (TNFalpha, IFN-γ, IL-2, IL-4, IL-5, IL-10 and IL-17) using (Polymerase Chain Reaction) techniques to analyze frozen genital tissues. Total RNA was isolated from the tissues using the QiagenRNeasy Mini Kit. RT-PCR products were generated by using the oligonucleotides or RT-PCR primers and RT-PCR conditions [12]. Cytokines were analyzed as per protocol using Qiagen one step RT-PCR kit. Gene expression was quantified by agarose gel electrophoresis.

2.6 Data Analysis

The Graph pad prism and SPSS version 20.0 statistical packages were used for the analysis of data obtained from the study.

3. RESULTS

The humoral immune response against *C. trachomatis* after genital infection was captured: antibody result infected was compared to naïve. The effect of caspase inhibitor on antibody response against *C. trachomatis* was captured and the results of Z-VAD-FMK were compared to non-treated group.

3.1 Antibody –ELISA Results

3.1.1 Electrophoregram

The T cell-mediated immune response against *C. trachomatis* after genital infection was captured: cytokines result of infected was compared to naïve as shown in Table 2. Effect of caspase inhibitor on cytokine response against *C. trachomatis* was also captured and the result of Z-VAD-FMK group was compared to non-treated group as shown in Table 2.

4. DISCUSSION

Genital infection of female mice with *Chlamydia trachomatis* causes oviduct (fallopian tube) pathoslogies and infertility [9]. In this study Chlamydia-induced infertility manifests as a reduction in the number of pregnancies resulting from mating after infection and the mean number of viable embryos in the groups of mice. Pre-treatment of mice with certain caspase inhibitors have been shown to reduce Chlamydia-induced infertility [9]. Since adaptive immunity is important in controlling Chlamydial infection in mice, and anti-caspase has been shown to reduce the pathology from Chlamydia infection, we then studied the effect of anti-caspase treatment on anti-chlamydia immune response.

Following genital infection of mice with *C. trachomatis*, there was an increase in total antibody response (total IgG) against *C. trachomatis* in infected mice compared to naïve mice. This result infers that after genital infection with *C. trachomatis*, there was an increase in humoral response. This is because B-lymphocytes which were activated by antigen presenting cells (APCs), develop into plasma

cells which then produce antibodies such as immunoglobulins (Igs) of different isotypes that recognize chlamydia proteins. The dominant immunoglobulin isotype found in the cervico-vaginal fluid of the female genital tract is IgG rather than secretory IgA.

The antibody levels (IgG, IgG1, IgG2a and IgA) in Z-VAD-FMK treated group was significantly higher than non-treated group suggesting that the caspase inhibitor does not negatively affect humoral immune response against *C. trachomatis*. When the treatment with Z-VAD was divided into pre- and post-treatment groups; pre-treatment being the application of caspase inhibitors before infection, while the post treatment involves the application of the caspase inhibitor after infection with chlamydia. ELISA results (Figs. 2-5) showed a significantly higher amount of antibodies (IgG, IgG1, IgG2a and IgA) was produced in the mice that were pre-treated with Z-VAD-FMK before infection with *C. trachomatis* compared to mice post treated with Z-VAD-FMK after *C. trachomatis* infection. Z-VAD-FMK is a cell-permeable pancaspase peptide inhibitor that irreversibly binds to the catalytic site of caspases, and inhibits caspase mediated apoptosis by preventing the processing of pro-caspases to their active forms. It can be inferred from the above result that Z-VAD-FMK administered before infection with *C. trachomatis,* increases the stimulation and proliferation of B cells thereby increasing the antibodies [6,7,13] which are higher in the pre-treated mice.

These results might imply that the higher amount of antibodies present in pre-treated mice would neutralize the antigen or directly destroying the pathogen; inactivating extracellular elementary bodies (EBs) [10,4]. Moreover B-lymphocytes can serve as APCs for T-lymphocytes. As a consequence, although antibodies can help in clearance of infection, their major role is in the enhancement of Th1 activation [13]. The results would therefore predict a higher T cell response against *C. trachomatis* after caspase inhibitor treatment.

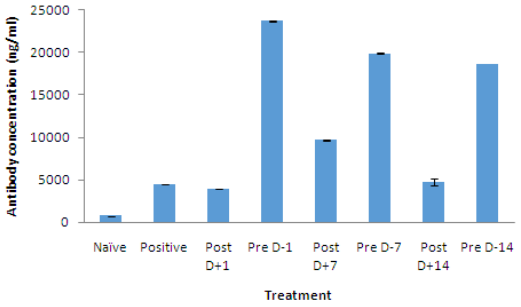

Fig. 2. Total IgG level in *C. trachomatis* infected mice treated with Z-VAD-FMK

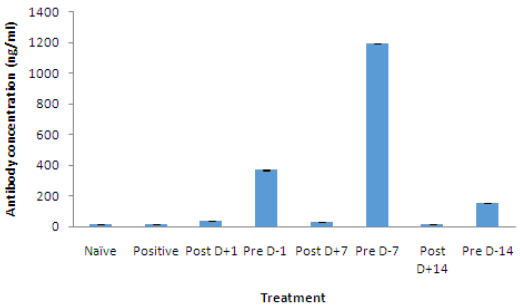

Fig. 3. IgG1 level in *C. trachomatis* infected mice treated with Z-VAD-FMK

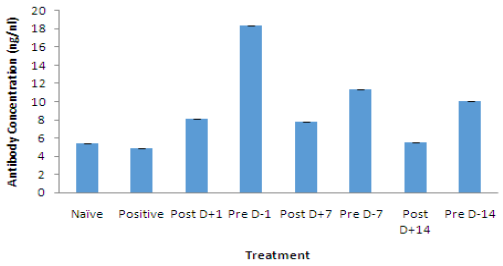

Fig. 4. IG2a level in *C. trachomatis* infected mice treated with Z-VAD-FMK

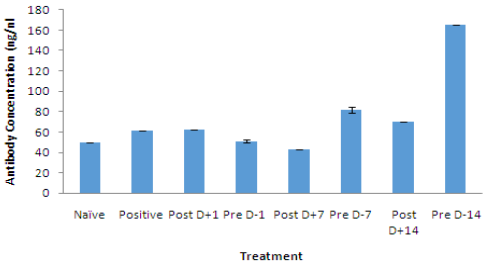

Fig. 5. IgA level in *C. trachomatis* infected mice treated with Z-VAD-FMK

IgG, IgG1, IG2a and IgA are different isotypes of immunoglobulin. Z-VAD-FMK (Fluoromethylketone) is a caspase inhibitor

Table 2. RT-PCR gene expression

	Normal uninfected mice at week 8	**Wild type mice infected with *C. trachomatis* at week 8 of infection**	**Wild type mice infected with *C. trachomatis*at week 8 of infection**	**Wild type mice that was given Z-FA-FMK (control) at week 8**	**Wild type mice that was given Z-VAD-FMK at week 8**	**Negative control, no RNA at week 8**
	Group (GP) 1	**Group (GP) 2**	**Group (GP) 3**	**Group (GP) 5**	**Group (GP) 6**	**Group (GP) 7**
Th1 Cytokines/ Proinflammatory						
TNF-alpha	-	†	††	†††	†††††	—
IFN-γ	—	†††	†	††	††††	—
IL-2	—	—	—	—	†	—
Proinflammatory						
IL-17	-	††	††	††	†††††	—
Th2 Cytokines/ anti-inflammatory						
IL5	—	—	—	—	†††	—
IL4	—	—	—	††	†††††	—
IL-10	—	—	—	†	†††	—

TNF-alpha	*Tumour necrosis factor alpha*
IFN-γ	*Interferon-gamma*
IL-2	*Interleukin-2*
IL-17	*Interleukin-17*
IL-5	*Interleukin-5*
IL-4	*Interleukin-4*
IL-10	*Interleukin-10*

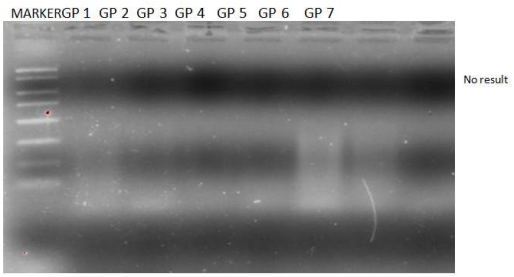

Fig. 6. Actin level

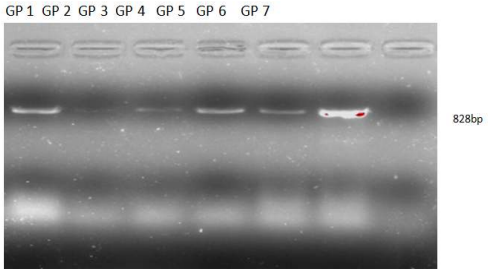

Fig. 7. TNF-alpha level

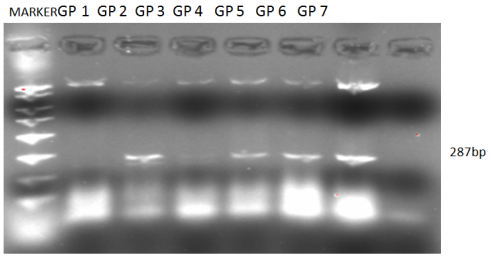

Fig. 8. IFN-y level

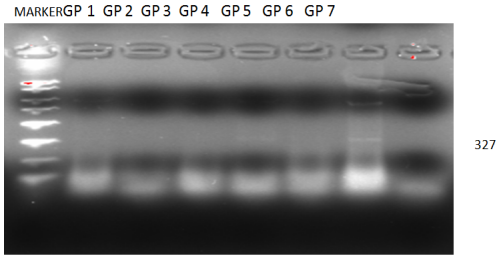

Fig. 9. IL-2 level

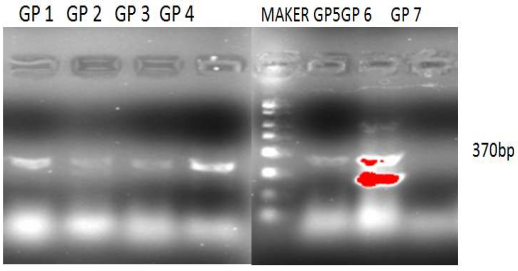

Fig. 10. IL-17 level

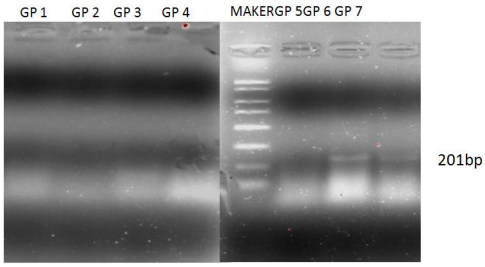

Fig. 11. IL-5 level

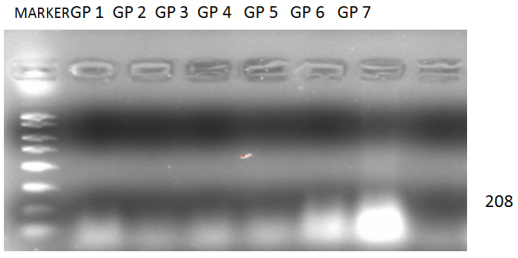

Fig. 12. IL-4 level

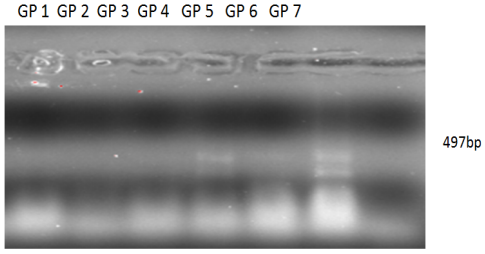

Fig. 13. IL-10 level

Figs. 6-13. The RT-PCR gene expression of the cytokines level in *C. trachomatis* infected mice treated with Z-VAD-FMK

The use of RT-PCR to measure T cell cytokines produced as a result of chlamydial activation of T cell-mediated immune response was also information (Table 2 and Figs. 6-13). This results showed an increase in pro-inflammatory cytokines (TNF-alpha, IFN-γ, IL2 and IL17) in the Z-VAD-FMK treated mice compared with the naïve group. There also appeared to be an increase in anti-inflammatory cytokines (IL5, IL4 and IL10) in Z-VAD-FMK treated mice compared to the naïve group. It can be inferred that T lymphocytes were activated by APCs and B

lymphocytes [9] and [11] leading to an increase in T cell-mediated immune response. The results indicated that the caspase inhibitor used in this study had no negative effect on T cell response against Chlamydia.

There was a significantly higher level of pro-inflammatory cytokines and anti-inflammatory cytokines in Z-VAD-FMK treated groups compared to untreated group, with the expression of these cytokines higher in the pre-treated group compared with the post-treated group. This corroborates the antibody ELISA results that showed high levels of anti-chlamydia antibodies in the pre-treatment group.

Taken together, the results showed an increased immune response in the presence of the caspase inhibitor, Z-VAD-FMK. Conceivably, immune cells activated against *C. trachomatis* were recruited to the site of infection and cytokines were produced with both Th1 and Th2 cytokines being produced, immune responses being turned on, although the Th1 cytokines were more prominent. Th1-type responses (characterized by the signature cytokine IFN-γ) play a role in the resolution of infection, whereas Th2-type responses involved in the humoral immunity is crucial for scarring [1]. The results from the cytokine expression could be putatively interpreted as having a balance in the Th1/Th2 cytokines, thus not leading to the normal disruption and pathogenesis of chlamydia infection, which resulted in normal phenotype of the oviduct leading to normal pregnancy in mice treated with this caspase inhibitors. The information provided in this study may be useful in vaccine production against Chlamydial infections.

5. CONCLUSION

The results from this study indicate that the caspase inhibitor, Z-VAD-FMK did not negatively affect humoral and T cell mediated immune responses against *C. trachomatis*. It can thus be inferred that from the results that Z-VAD-FMK increases the stimulation and proliferation of B cells due to the increased production of Th2 cytokines and thus can be corroborated in the ELISA results where we have shown high amount of antibodies produced in the mice that were treated with Z-VAD-FMK before infection with *C. trachomatis*. The use of caspase inhibitors in ameliorating destructive effect of infectious disease such as Chlamydia is novel, this could be used in the treatment of women infected with Chlamydia and this would lead to a reduction in the formation of hydrocoels, salpigitis, ectopic pregnancies which occur from secondary infection with Chlamydia.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It was carried out in accordance with APA 2010.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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