

To the Preeclampsia Foundation:

Just wanted to share with you the good news. My project, that was of course, funded by the Foundation, resulted in a poster presentation this year (2003) at the Society for Maternal Fetal Medicine Conference. In addition, that project has turned into a publication that has been accepted by the American Journal of Ob/Gyn, and should be coming out in the October issue. It will be entitled "Differential Expression of TcR-CD3 Zeta as Evidence for Altered Immunoregulation in Preeclamptic vs. Normotensive Women." Attached is a copy.

I am now graduating from the University of North Carolina, and moving on to practice in Phoenix, AZ, though I do hope to continue my work in the zeta chain expression subject area, and build upon what I started through the Preeclampsia Foundation's help.

Thank you again for your support. I hope my efforts will help represent the good work and intentions that the Foundation puts forth.

Sincerely,
Garrett Lam, MD

Differential Expression of TcR-CD3 Zeta as Evidence for Altered Immunoregulation in Preeclamptic vs. Normotensive Women

Garrett K. Lam, MD¹
Paul W. Whitecar, MD²
Susan Orton, PhD³
Kim A. Boggess, MD¹
Douglas D. Taylor, PhD⁴

¹ Division of Maternal Fetal Medicine, Dept of Obstetrics and Gynecology, University of North Carolina School of Medicine

² Dept of Obstetrics and Gynecology, Womack Army Medical Center

³ Dept of Immunology, University of North Carolina School of Medicine

⁴ Division of Gynecologic Oncology, Dept of Obstetrics and Gynecology, University of Louisville School of Medicine

Address Correspondence and Reprints to:

Garrett Lam, MD
214 MacNider Building
CB7516
Chapel Hill, NC 27703
Phone: (919) 966-1601
Fax: (919) 966-6377

E-mail: garrett_lam@med.unc.edu

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Condensation

Analysis of immunologic parameters indicates that preeclampsia is characterized by decreased suppression of CD3-zeta expression, inferring increased T-cell function compared to normotensive pregnancies.

ABSTRACT

Differential Expression of TcR-CD3 Zeta as Evidence for Altered Immunoregulation in Preeclamptic vs. Normotensive Women

Garrett K. Lam, MD, Paul W. Whitecar, MD, Susan Orton, PhD, Kim A. Boggess, MD,
Douglas D. Taylor, PhD

Objective: To exhibit and quantify the difference in modulation of CD3-zeta protein (an integral component of the T-cell receptor) in preeclamptic and normotensive women.

Study Design: Serum was collected from 10 preeclamptic and 10 normotensive women ≥ 37 weeks gestation upon admission. Jurkat E-61 cells were incubated with the sera (20% volume to volume) and analyzed via western immunoblot using mouse monoclonal CD3-zeta antibody. Enhanced chemiluminescence and densitometry were used to qualitatively measure zeta expression of the cells. A de novo flow cytometry assay was developed to quantify the difference in CD3 zeta expression of these cells. Comparisons were done by t-test ($p < 0.05$ was significant).

Results: Preeclamptic patient sera produced a 2.4-fold increase in CD3-zeta expression than normotensive patients on Western blot ($p < 0.01$). Flow cytometry showed that preeclamptic sera had a 1.4 fold higher expression of CD3-zeta compared to normotensive patients ($p < .0003$).

Conclusion: TcR/CD3-zeta expression is normally suppressed in pregnancy. Loss of this suppression occurs in preeclamptics, implying increased T cell function.

Key words: TcR-CD3 zeta protein, T-cell function, preeclampsia, **INTRODUCTION**

Preeclampsia occurs in approximately 7-12% of all pregnancies, and remains a highly malignant condition of pregnancy. Worldwide, preeclampsia affects 3 million women annually, resulting in an estimated 76,000 maternal deaths. Women in third world countries are particularly at risk, with mortality rates reported as high as 8-14%.¹ According to United States registry data, from 1979 and 1992, 790 of 4024 pregnancy-related deaths at 20 weeks or more gestation were due to preeclampsia or eclampsia.² Preeclampsia is also associated with a five-fold increase in perinatal mortality. Despite such notoriety, the etiology of preeclampsia remains unknown.

Maternal immunologic function appears to be altered in normal pregnancy. For example, infection from intracellular pathogens (viz., tuberculosis and malaria) and viruses (i.e. hepatitis, herpes simplex) are more severe.³ This implies that cell-mediated (also known as T helper cell type 1 or Th1) immunity is depressed in pregnancy.

Prior work by Bedenicki et al has illustrated that suppression of Th1 type immunity in pregnancy is linked to down-regulation of the CD3-zeta chain complex.⁴ CD3-zeta is the intramembranous part of the T-cell receptor that is critical for T-cell proliferation. Secretion of IL-2, a Th1 cytokine that stimulates T cell (along with B cell and natural killer cell) growth, is mediated through this complex.⁵ Thus, one would expect that successful pregnancy would involve decreased CD3-zeta expression to inhibit cell-mediated/Th1 immunity to protect the fetal allograft from rejection.

Preeclampsia, meanwhile, may be considered as “an excessive maternal inflammatory response to pregnancy” (Redman, 1999).⁶ Recent publications have shown that cytokine expression is upregulated in preeclamptic pregnancies compared to normotensive pregnancy controls. For example, Saito et al⁷ illustrated that preeclamptic patients exhibited significantly higher Th1 type cytokine production than normotensive patients. Sunder-Plassman et al⁸ further showed that serum levels of IL-2 are increased in preeclamptics.

Our group has previously presented data which supports the possibility of increased T-cell function in preeclampsia. We previously obtained midtrimester sera from women who eventually developed preeclampsia at term, and examined its effects on CD3-zeta expression in an in vitro T-cell model. These results were compared with results obtained from similar experiments done with midtrimester sera obtained from normotensive pregnant women. This study supported the notion that showed that preeclamptic sera appeared to have lower levels of CD3-zeta suppression and increased IL-2 expression thcompared to normotensive controls, implying a “hyperimmune condition.”⁹

We now seek to characterize and quantify CD3-zeta chain expression in preeclamptic womencompared to normotensive women. Given that preeclampsia is a hyperinflammatory state of pregnancy, CD3-zeta expression should be increased in preeclamptic women compared to their normotensivecounterparts.

MATERIALS AND METHODS

This study was approved by the human Investigational Review Board of the University of North Carolina. A power calculation was performed which determined that, in order to show a 30% difference in zeta protein expression between preeclamptic and normotensive women, a total sample size of 16 patients would be needed to achieve 90% power with an alpha of 0.05.

Patient-derived materials:

Maternal serum was collected from 20 women at term gestation (≥ 37 weeks at presentation) upon presentation to the Labor and Delivery unit at the University of North Carolina Hospitals. All samples were assessed blinded. Ten samples were collected from women with preeclampsia defined by the National High Blood Pressure Working Group¹⁰ as new onset systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 with at least 1+ proteinuria on semi quantitative dipstick. These patients also had 12-hour urine collections for protein >165 mg/12 hrs. Women with a history of chronic hypertension, active renal disease, diabetes and tobacco use during pregnancy were excluded. Ten control samples were collected from normotensive women with similarly benign medical histories who delivered uncomplicated pregnancies. Ethnicity, chronologic age and gestational age were recorded. Demographic variables were analyzed via a two-tailed Student t-test.

TcR/CD3-zeta Expression:

Jurkat E-61 cells (American Type Culture Collection, Rockville, MD), a human T-cell lymphoma with a functional TcR/CD3 complex capable of synthesizing IL-2, were utilized as an

in vitro assay for lymphocyte modulation by sera from study patients. Jurkat cells were grown in RPMI 1640 medium supplemented with 0.1mM nonessential amino acids, 1mM sodium pyruvate, 200mM L-glutamate, 100µg/ml streptomycin and 100 IU/ml penicillin in a humidified 5% CO₂ chamber at 37°C. Cell viability was evaluated by trypan blue exclusion. All cultures utilized for this study were >95% viable. Initial analysis of CD3/TcR-zeta expression was performed in a blinded fashion. Viable Jurkat cells (10⁶ cells/ml) were incubated in a medium supplemented with 20% patient serum for 4 days. After 4 days, the cells were centrifuged, the cell pellet washed and subsequently lysed using 50mM HEPES, pH 7.2, 150mM NaCl, 5mM EDTA, 1mM Na-orthovanadate, 2.5% Triton X-100, 200µg/ml trypsin/chymotrypsin inhibitor, 200µg/ml chymostatin and 2mM PMSF. The cell lysate was assayed for protein by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA).

The modulation of CD3-zeta between the two study groups was analyzed by two methods. Qualitative differences in zeta expression were assessed by western immunoblot using a 15% SDS-PAGE gel. Twenty-five µg of protein from each fragment isolate was applied per lane of a 12.5% SDS-PAGE gel. The proteins were electrophoretically separated by the method of Laemmli¹¹ and analyzed by western immunoblot as described by Brown et al.¹² The blots were blocked with 10% non-fat dry milk and probed overnight at 4°C in the same buffer with mouse anti-CD3-zeta monoclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) as the primary antibody. After this incubation, the membranes were washed three times in 0.1% Tween 20 in TBS for 15 min. The blots were then incubated in blocking buffer with rabbit anti-mouse IgG with horseradish peroxidase (Sigma Chemical Co., St. Louis, MO) for 45 min. at room temperature. These immunoblots were washed three times in 0.1% Tween 20 in PBS, after

which, bound complexes were visualized by enhanced chemiluminescence (ECL, Amersham Life Sciences, Arlington Heights, IL-Figure 1) and quantitated by densitometry (Un-Scan-It Software, Silk Scientific Corp., Orem, UT).

Quantitative differences in CD3 zeta expression were assessed using a de novo intracellular flow cytometry protocol. Jurkat cells previously incubated with 20% patient sera for 4 days were washed once and resuspended to original cell concentration with PBS containing 1% bovine serum albumin (BSA). Caltag's Fix and Perm kit (Caltag Labs, Inc., Burlingame, CA) was used to fix and permeabilize the cell membranes to facilitate intracellular staining with anti-CD3 zeta antibody. Following a 15 minute incubation with fixative and washing, 50 μ l of either phycoerythrin (PE)-labeled mouse monoclonal anti-CD3 zeta. (Santa Cruz Biotechnologies) or PE-labeled negative isotype control antibody (Beckman Coulter, Immunotech, Miami, FL) along with permeabilization reagent was incubated with the cells for 30 minutes at room temperature. The cells were washed twice and analyzed on a FACScan cytometer (Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA). Single laser analysis was carried out using an air-cooled 15-mW argon laser, operating at 488 nm. Instrument setup was performed daily using Calibrite beads (BD Biosciences, San Jose, CA) and FACSCComp software. For these set of experiments forward and side scatter were collected on a linear log scale respectively. A single gate(R1) was established in order to analyze viable cells by flow cytometry. This cell population specified by the gate was chosen for specific scatter properties. Ten thousand events of interest (R1) were collected as list mode data and analyzed using CellQuest software. The PE-labeled isotype negative control was run to determine non-specific binding of the anti-CD3 zeta antibody. To standardize the PE fluorescence signal, SPHEROTm Rainbow Calibration

beads (Sperotech, Inc., Libertyville, IL) were used to adjust the PMT voltages to target the mean peak channels for PE between 610-614. Using the same instrument settings, samples and PE-labeled QuantiBRITE beads (BD Biosciences, San Jose, CA) were run. A standard curve was constructed by plotting the known number of PE molecules present on the QuantiBrite beads against the mean peak channel number for each of the beads using QuantiQuest software (BD Biosciences). Regression analysis was then used to determine the number of PE molecules bound per cell for the unknown samples. Results are reported as antibodies bound per cell .

Relative absorbance determinations of the densitometry values of zeta expression were performed twice for each population, and the mean and standard deviations were calculated. Band intensity between preeclamptics and controls were compared by Student's t-test ($p < 0.05$ was significant). Likewise, the mean and standard deviations for ABC differences were calculated, and were also compared using the Student's T-test assuming unequal variances. Tests with $p < 0.05$ were considered statistically significant. Statistical analysis was performed using Stata version 6.0 (College Station, TX).

RESULTS

Patient characteristics:

All women enrolled in the study were between the ages of 18-36. By study design, women were enrolled on a rolling basis if eligibility criteria were met, thus, we were unable to match cases and controls by ethnicity and gestational age. Maternal demographics are presented in Table 1.

Preeclamptic women were younger than normotensive women (mean age 22.3 years vs 30.4 years), but were of similar gestational age and racial distribution.

TcR/CD3 zeta expression via Western blot:

Mean densitometry measurements indicating CD3-zeta expression were higher in preeclamptic women compared to normotensive women (mean 71660, range 48000 to 91400 absorbance units, vs mean 43800, range 29300 to 64300 absorbance units, $p < 0.0002$ -Figure 2).

CD3 zeta expression using quantitative flow cytometry:

Quantification of events counted by flow cytometry, indicating number of antibodies bound per cell, showed that preeclamptic women had higher CD3-zeta expression than normotensive women (mean 10274 ± 467 events, vs 7340 ± 384 events, $P < 0.0003$ -figure 3).

COMMENT

In normal pregnancy, there are several alterations in the maternal immune system that allow immune tolerance of the fetus. Among these is the possibility of a change in the ratio of Th2 to Th1 type immunity, wherein Th1 cells that are responsible for cell mediated immunity function are downregulated.¹² This change may be mediated through suppression of CD3-zeta, which would subsequently impair T-cell function. Suppression of TcR-CD3 zeta expression in maternal serum has been demonstrated in normal pregnancies, with suppression increasing as pregnancy progresses.⁵

If pregnancy represents a relatively immune suppressed state, then perhaps preeclampsia represents the opposite end of the spectrum. Th1 lymphocytes produce pro-inflammatory cytokines, among them IL-2, TNF- β and interferon-gamma. IL-2 secretion, a cytokine that activates lymphocytes, natural killer cells and macrophages, has previously been shown to increase in preeclamptic patients versus those with normal pregnancies.^{7,8} Whitecar et al⁹ presented data on sera collected from women at mid-trimester who eventually became preeclamptic, that appeared to increase Jurkat cell expression of TcR-CD3 zeta and production of IL-2.

Our study attempts to improve upon previous work by first refining the study population by prospectively enrolling women at term who were nulliparous, and presented with strictly defined symptoms of preeclampsia. Possible confounding co-morbidities such as tobacco use (which could conceivably induce proinflammatory cytokine production) and chronic hypertension, diabetes, or renal disease (which could cause proteinuria and elevations in blood pressure not entirely reflective of preeclampsia) were excluded. Our population demographics were, in general, representative of a population of women at risk for preeclampsia (minority ethnic extraction and young maternal age).

This study represents the first description of a protocol that directly quantifies the amount of zeta production induced in Jurkat cells. The use of flow cytometry enables us to accurately characterize the statistically significant difference in zeta chain production induced by sera from preeclamptic subjects in comparison to the normotensive women. Using both our qualitative and quantitative methods, we have shown that sera from normotensive women induced suppression of zeta chain production in Jurkat lymphocytes, a behavior that would be expected from a normal

pregnancy. However, sera taken from term preeclamptic women resulted in a significantly increased production of TcR-CD3 zeta, which would support the idea that a hyperimmune state exists with preeclampsia.

In conclusion, preeclampsia is likely the end result of altered relationship between mother and fetus. Perhaps preeclampsia represents a shift in the maternal immune system that changes maternal tolerance to a hemi-allogeneic fetus. Our study has confirmed a possible role for modification of the Th1/Th2 ratio by demonstrating a lack of suppression of TcR/CD3-zeta expression in a strictly defined population of preeclamptic women. There may be some serum factor, as yet to be identified, that exists in normal pregnancies and leads to the down-regulation of CD3-zeta. Presumably, this factor is lacking in pregnancies complicated by preeclampsia. One of our goals for future studies would be to identify the factor that serves as an upstream regulator of TcR-CD3 zeta expression. We also plan to refine the flow cytometry technique in order to directly measure CD3-zeta expression in vivo. It is our hope that further work on the modulation of CD3-zeta expression will help solve the enigma of preeclampsia.

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Table 1

Parameter	Preeclamptics (n=10)	Normotensives (n=10)	P value ¹
Age (years)	22.3	30.4	P<0.02
Gestational Age (weeks)	38.3	39.2	NS
Ethnicity (#minorities ² /total)	8/10	5/10	NS

¹Student's T-test, significant for p<0.05

²Minorities=Black and Hispanic ethnicities

Figure 1

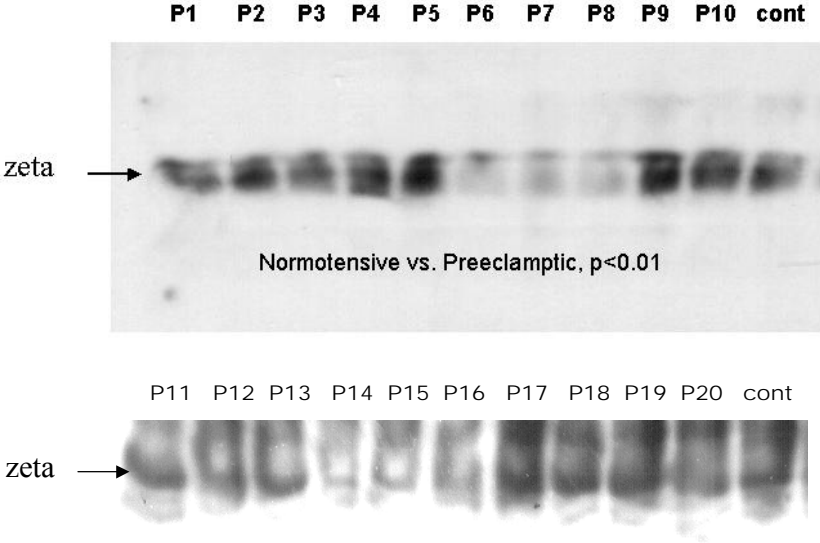


Figure 2

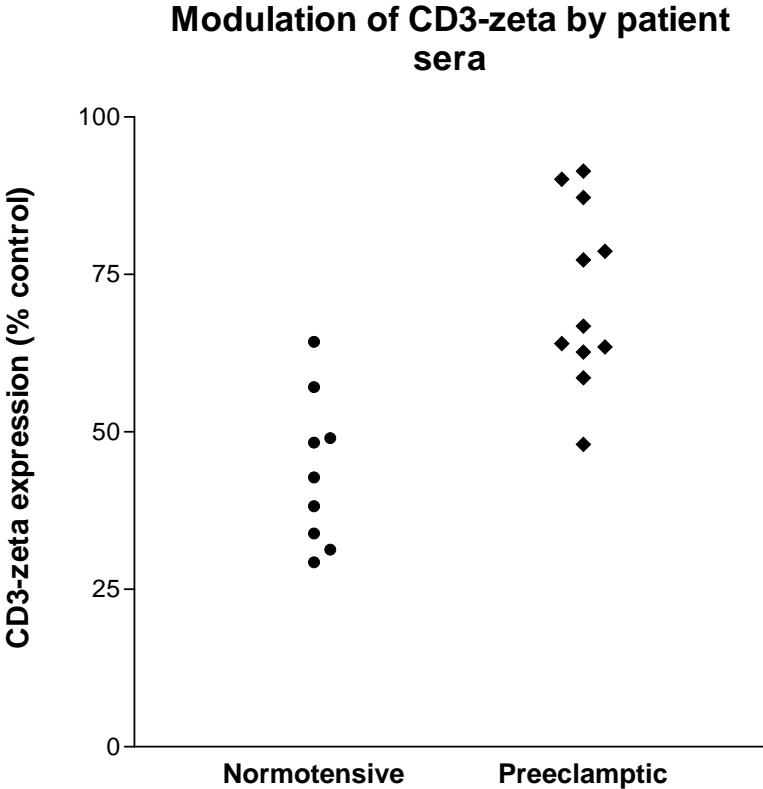
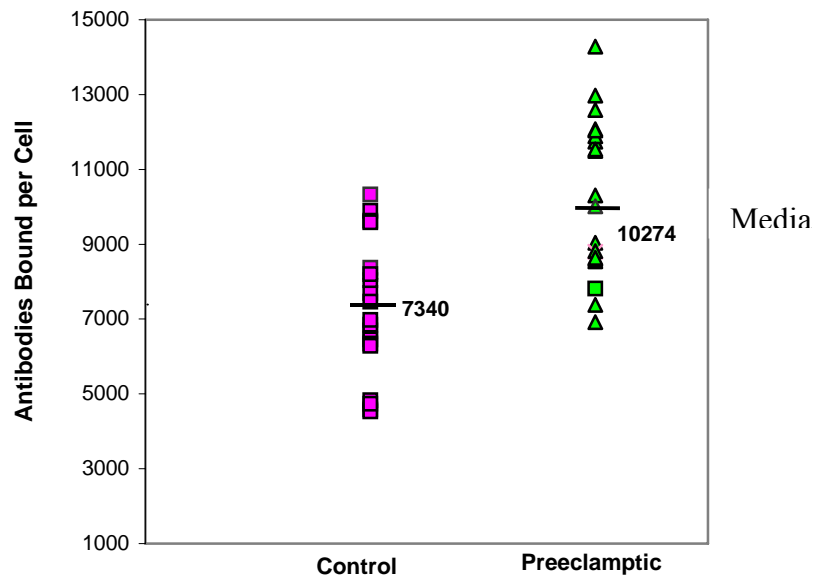


Figure 3

Quantitative CD3 zeta Expression in Jurkat Cells Using Flow Cytometry



Legend

Table 1: Comparison of Study and Control Patient Demographics

Figure 1: Enhanced Chemiluminescence of Western Blot for TcR-CD3 Zeta Protein from Jurkat

E-61 Cells after Incubation with Study and Control Patient Sera

Preeclamptic patients represented by samples 1,2,3,5,9,11,13,17,18,19

Normotensive patients represented by samples: 4,6,7,8,10,12,14,15,16,20

Figure 2: Modulation of CD3-Zeta by Patient Sera

Figure 3: Quantitative CD3-Zeta Expression in Jurkat Cells Using Flow Cytometry