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## **Intraperitoneal Injection of a Monoclonal $\alpha$ CD3 $\epsilon$ Antibody Induces Preterm Labor and Birth**

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**PROBLEM**

Activated/effector T cells seem to play a role in the pathological inflammation associated with preterm labor. The aim of this study was to determine whether *in vivo* T cell activation, via a monoclonal  $\alpha$ CD3 $\epsilon$  antibody, induces preterm labor and birth.

**METHOD OF STUDY**

Pregnant B6 mice were intraperitoneally injected with  $\alpha$ CD3 $\epsilon$  or its isotype control. The gestational age, the rates of preterm birth and pup mortality at birth, as well as the fetal heart rate and umbilical artery pulsatility index were determined.

**RESULTS**

Injection of  $\alpha$ CD3 $\epsilon$  led to preterm labor/birth [ $\alpha$ CD3 $\epsilon$  83  $\pm$  16.97% (10/12) vs. isotype 0% (0/8)], and increased the rate of pup mortality at birth [ $\alpha$ CD3 $\epsilon$  87.30  $\pm$  8.95% (77/85) vs. isotype 4.91  $\pm$  4.34% (3/59)]. In addition, injection of  $\alpha$ CD3 $\epsilon$  decreased the fetal heart

rate and increased the umbilical artery pulsatility index when compared to isotype controls.

## CONCLUSION

Intraperitoneal administration of  $\alpha$ CD3 $\epsilon$  in late gestation induces preterm labor and birth.

**KEYWORDS:** adaptive immunity, cytokines, maternal-fetal rejection, mouse, parturition, pregnancy, T cells

## INTRODUCTION

Preterm birth, delivery before 37 weeks of gestation, is the leading cause of perinatal morbidity and mortality worldwide. Approximately 70% of all preterm births occur after spontaneous preterm labor<sup>1</sup>, a syndrome of multiple etiologies<sup>2</sup>. Pathological inflammation is implicated in the process of preterm parturition<sup>3-5</sup>, and can result from the activation of innate<sup>6-12</sup> or adaptive immunity<sup>13, 14</sup>. Among adaptive immune cells, T cells are implicated in the mechanisms that lead to spontaneous labor at term<sup>15-17</sup> and spontaneous preterm labor<sup>13, 14, 18</sup>.

T cells are adaptive immune cells that are critical for antigen specific immunity as well as defense against future infections. The defining feature of T cells is the T cell receptor (TCR), which allows them to perform most of their antigen-specific functions through interactions with MHC class I and class II molecules. T cell subsets include: 1) CD4+ T helper (Th) cells, which respond to exogenous antigens presented through MHC class II signaling<sup>19-23</sup>; and 2) CD8+ cytotoxic T cells or CTLs, which are involved in the lysis of aberrant cells and respond to endogenous antigens or self-recognition through MHC class I signaling<sup>19, 20, 23</sup>. Discrimination of self and non-self<sup>24</sup>, along with the concept of tolerance<sup>25-27</sup>, are two of the most clinically important aspects of T cell functionality, as even slight errors in either process can lead to diseases such as autoimmune disorders. T cells are activated through the engagement of the TCR and co-stimulation<sup>28</sup>. Upon activation, effector T cells secrete cytokines which can promote T cell proliferation and the activation of T cell-dependent B cells, as well as regulate the activity of innate immune cells such as macrophages<sup>28</sup>. *In vivo* T cell activation is achieved by administering low concentrations (4-10 $\mu$ g) of a monoclonal  $\alpha$ CD3 $\epsilon$  antibody (e.g. clone 145-2C11)<sup>29, 30</sup>. This antibody recognizes the CD3 $\epsilon$  molecule and activates

T cells in the absence of antigen, since it evades the TCR antigen-specific recognition mechanism<sup>31, 32</sup>. Herein, we hypothesized that the administration of a monoclonal  $\alpha$ CD3 $\epsilon$  antibody (clone 145-2C11) in late gestation will cause pathological inflammation by initiating innate and adaptive immune responses which, in turn, could lead to preterm labor and birth.

The aim of this study was to determine whether *in vivo* T cell activation via a monoclonal  $\alpha$ CD3 $\epsilon$  antibody induces preterm labor and birth. Also, we examined whether administration of this antibody would cause fetal death or fetal compromise using Doppler ultrasound.

## **MATERIALS AND METHODS**

### **Animals**

C57BL/6J (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and bred in the animal care facility at the C.S. Mott Center for Human Growth and Development at Wayne State University, Detroit, Michigan, USA. Mice were housed under a circadian cycle (light: dark=12:12 h). Eight- to 12-week-old females were mated with males of proven fertility. Females were examined daily between 8:00 a.m. and 9:00 a.m. for the presence of a vaginal plug, which indicated 0.5 days *post coitum* (dpc). Upon observation of a vaginal plug, females were housed separately from males, their weight was monitored, and a gain of two or more grams by 12.5 dpc confirmed pregnancy. Procedures were approved by the Institutional Animal Care and Use Committee at Wayne State University (Protocol No. A 09-08-12).

### **Intraperitoneal administration of anti-mouse CD3 $\epsilon$**

Pregnant B6 mice were intraperitoneally injected with 10 $\mu$ g of a purified anti-mouse CD3 $\epsilon$  ( $\alpha$ CD3 $\epsilon$ ) (BD Biosciences, San Jose, CA, USA, Clone 145-2C11; n=12) dissolved in 200 $\mu$ L of sterile 1X phosphate-buffered saline (PBS) on 16.5 dpc. Controls were injected with 10 $\mu$ g of isotype (IgG1  $\kappa$  Isotype; BD Biosciences, Clone A19-3; n=8) dissolved in 200 $\mu$ L of PBS on 16.5 dpc. Following injection, mice were monitored using a video camera with an infrared light (Sony Corporation, Tokyo, Japan) until delivery.

### **Outcome variables**

Preterm labor/birth was defined as delivery occurring before 18.0 dpc, and its rate was represented by the percentage of females delivering preterm among those delivering at term ( $19.5 \pm 0.5$  dpc). Gestational age was defined as the time elapsed from the detection of the vaginal plug (0.5 dpc) through the delivery of the first pup. The rate of pup mortality at birth was defined as the percentage of pups found dead among the total litter size.

### ***In vivo* imaging by ultrasound**

Pregnant B6 mice were intraperitoneally injected with  $\alpha$ CD3 $\epsilon$  or isotype on 16.5 dpc, as previously described<sup>33, 34</sup> (n=12-13 each). Sixteen hours post-injection (prior to preterm labor/birth in mice injected with  $\alpha$ CD3 $\epsilon$ ), mice were anesthetized by inhalation of 2-3% of isoflurane (Aerrane, Baxter Healthcare Corporation, Deerfield, IL, USA) and 1-2 L/min of oxygen in an induction chamber. Anesthesia was maintained with a mixture of 1.5-2% of isoflurane and 1.5-2 L/min of oxygen. Mice were positioned on a heated platform and stabilized using adhesive tape. Fur was removed from the abdomen and thorax following the application of Nair cream (Church & Dwight Co., Inc., Ewing, NJ, USA) to those areas. Body temperature was maintained at  $37 \pm 1$  °C and monitored using a rectal probe. Respiratory and heart rates were monitored by electrodes embedded in the heated platform. An ultrasound probe was fixed and mobilized with a mechanical holder, and the transducer was slowly moved toward the abdomen. Fetal heart rate and umbilical artery pulsatility index (PI) were examined with the 55MHz linear ultrasound probe (VisualSonics Inc., Toronto, ON, Canada). Umbilical artery PI was calculated using the following formula:  $PI = (\text{systolic velocity} - \text{diastolic velocity} / \text{mean velocity})$ . Ultrasound signals were processed, displayed, and stored using the Vevo Imaging Station (VisualSonics Inc). Following ultrasound, females were placed under a heat lamp for recovery, which occurred 10-20 min after heating.

## Statistical Analysis

Statistical analyses were performed using SPSS, Version 19.0 (IBM Corporation, Armonk, NY, USA). The following tests were performed to compare differences between the groups: a Fisher's exact test for the rates of preterm labor/birth, a Mann-Whitney U-test for gestational age, a logistic regression model for the rates of pup mortality at birth, and T-tests for fetal heart rate and umbilical artery pulsatility index. A p value of 0.05 was considered statistically significant. When proportions are displayed, percentages and 95% confidence intervals are shown. Medians are shown with the interquartile range (IQR) and means are shown with the standard error of the mean (SEM).

## RESULTS

The frequency of preterm labor/birth after an intraperitoneal injection of  $\alpha$ CD3 $\epsilon$  was higher than following an intraperitoneal injection of isotype [ $\alpha$ CD3 $\epsilon$  83  $\pm$  16.97% (10/12) vs. isotype 0% (0/8);  $p < 0.0001$ ; Figure 1A]. Pregnant mice injected with  $\alpha$ CD3 $\epsilon$  had a shorter gestational age than those injected with isotype [ $\alpha$ CD3 $\epsilon$  17.51 dpc (IQR = 17.46-17.59 dpc) vs. isotype 19.19 dpc (IQR = 19.03-19.28 dpc);  $p = 0.002$ ; Figure 1B]. Intraperitoneal injection of  $\alpha$ CD3 $\epsilon$  was also associated with an increased rate of pup mortality at birth [ $\alpha$ CD3 $\epsilon$  87.30  $\pm$  8.95% (77/85) vs. isotype 4.91  $\pm$  4.34% (3/59);  $p < 0.0001$ ; Figure 1C].

Most of the dams injected with  $\alpha$ CD3 $\epsilon$  delivered premature non-viable pups (Figure 1C). We then investigated whether T cell activation was causing fetal death (i.e. fetuses without a heartbeat) or fetal compromise (i.e. fetuses with abnormal umbilical artery velocimetry and fetal heart rate<sup>35, 36</sup>). Therefore, Doppler ultrasound was performed (Figures 2A&2B) prior to preterm labor/birth in mice injected with  $\alpha$ CD3 $\epsilon$  or matched-time isotype controls. Fetuses from dams injected with  $\alpha$ CD3 $\epsilon$  were viable as a heartbeat was detected (Figure 2A). However, these fetuses were bradycardic when compared to controls [ $\alpha$ CD3 $\epsilon$  104.32 bpm (SEM  $\pm$  4.11 bpm;  $n = 88$ ) vs. isotype 154.69 bpm (SEM  $\pm$  3.54 bpm;  $n = 82$ );  $p < 0.0001$ ; Figure 2A]. Figure 2B shows how Doppler ultrasound is used to determine the blood flow through the umbilical artery. Fetuses

from dams injected with  $\alpha$ CD3 $\epsilon$  had an increased umbilical artery pulsatility index (PI) when compared to controls [ $\alpha$ CD3 $\epsilon$  1.83 PI (SEM  $\pm$  0.01 PI; n=87) vs. isotype 1.74 PI (SEM  $\pm$  0.01 PI; n=82); p=0.037; Figure 2B]. Together, these data demonstrated that, although pups from dams injected with  $\alpha$ CD3 $\epsilon$  did not die in the uterus, their health was compromised before birth.

## DISCUSSION

T cells have been implicated in the mechanisms that lead to spontaneous labor at term<sup>15-17</sup> and spontaneous preterm labor<sup>13, 14, 18</sup>. In the study herein, we demonstrated that the intraperitoneal injection of a monoclonal  $\alpha$ CD3 $\epsilon$  antibody induces preterm labor and birth. Administration of this antibody causes a massive systemic release of several T-cell derived cytokines such as tumor necrosis factor (TNF)- $\alpha$ , interferon (IFN)- $\gamma$ , interleukin (IL)-2 and IL-3<sup>37</sup>. These data suggest that T cell activation causes a systemic inflammatory response in the mother leading to preterm labor and birth.

Activated/effector CD8+ T cells or CTLs, and at a lesser extent CD4+ T cells, are observed in chronic inflammatory lesions of the placenta such as villitis of unknown etiology (VUE)<sup>38-40</sup>. CTLs are also abundant in systemic circulation<sup>13</sup>, endometrium<sup>41</sup>, ectocervix<sup>42</sup>, and in the chorioamniotic membranes of patients with chronic chorioamnionitis<sup>43</sup>, the most common placental lesion in late spontaneous preterm birth<sup>44</sup>. These cytotoxic T cells induce trophoblast apoptosis and damage the integrity of the chorioamniotic membranes<sup>14, 43</sup> which, in turn, may induce the premature rupture of these tissues and consequently lead to labor. Activated/effector T cells also mediate allograft rejection; indeed, both VUE and chronic chorioamnionitis are considered histopathologic manifestations of T-cell mediated rejection of the semi-allograft fetus<sup>14</sup>. All together, these data led us to propose that *in vivo* T cell activation represents a preterm birth model of maternal-fetal T-cell mediated rejection.

*In vivo* T cell activation caused fetal compromise by inducing bradycardia and an altered umbilical artery pulsatility index. This finding is consistent with two facts: 1) VUE is associated with an abnormal Doppler velocimetry of the umbilical artery<sup>45</sup>; and 2) chronic chorioamnionitis is associated with fetal death<sup>46</sup>. The negative effects of T cell activation on the fetal heart rate are most likely mediated by TNF- $\alpha$  and IL-2 (T cell

cytokines), which induce cardiomyopathy<sup>47, 48</sup>. Taken together, these data suggest that *in vivo* T cell activation induces fetal compromise by causing a fetal inflammatory response syndrome (FIRS), in which maternal-fetal rejection may be the mechanism of disease (i.e. FIRS type 2)<sup>14</sup>.

In summary, the study herein provides evidence that activation of maternal T cells, via a monoclonal  $\alpha$ CD3 $\epsilon$  antibody, induces fetal compromise and the premature expulsion of the semi-allograft fetus.

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## DECLARATION OF INTEREST STATEMENT

The authors report no declarations of interest.

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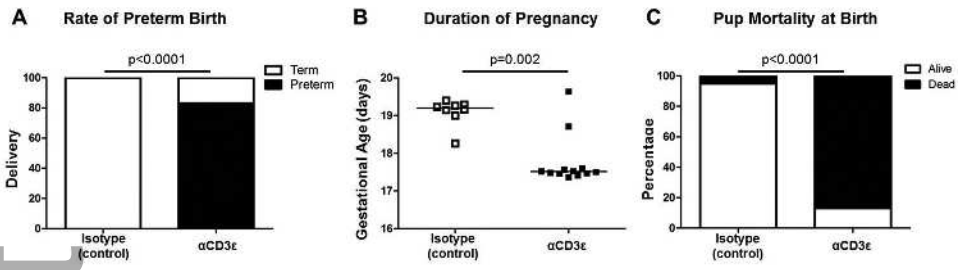
## FIGURE LEGENDS

Figure 1. Intraperitoneal injection of a monoclonal  $\alpha$ CD3 $\epsilon$  antibody. Pregnant B6 mice were intraperitoneally injected with a monoclonal  $\alpha$ CD3 $\epsilon$  antibody (10 $\mu$ g dissolved in 200 $\mu$ L of sterile PBS; n=12) on 16.5 days *post coitum* (dpc). Control mice were injected

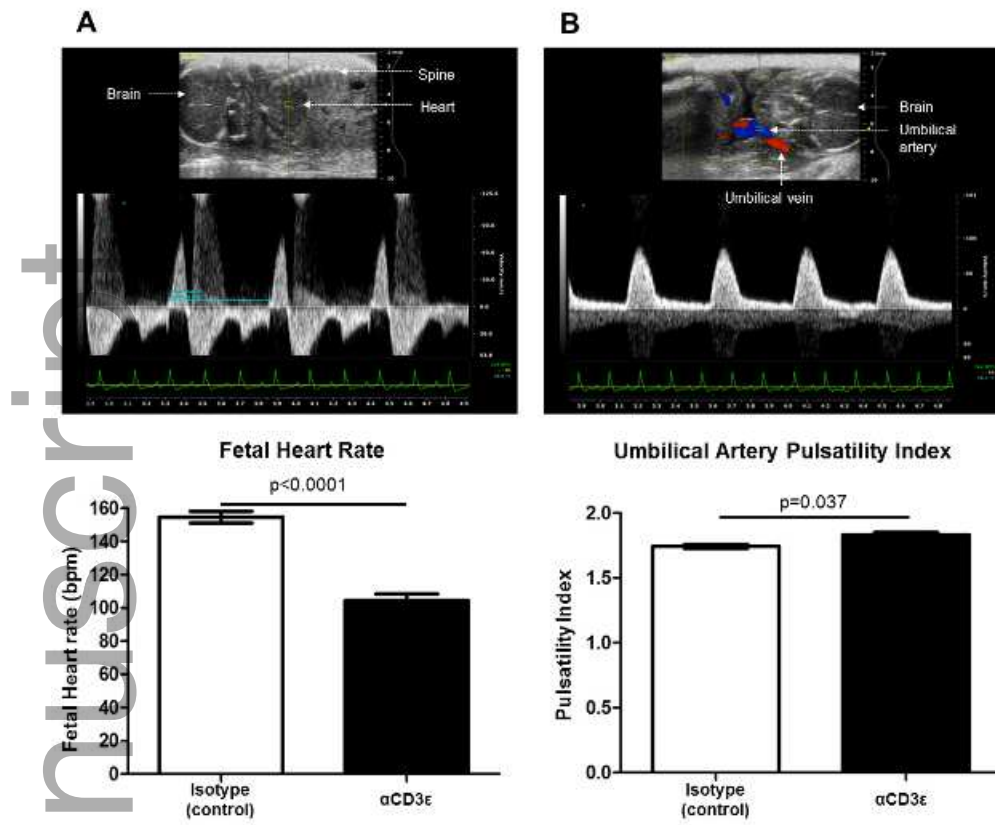
with an isotype (10 $\mu$ g dissolved in 200 $\mu$ L of sterile PBS; n=8) on 16.5 dpc. The rate of preterm labor/birth (A), gestational age (B), and rate of pup mortality at birth (C) are displayed.

Figure 2. *In vivo* imaging by Doppler ultrasound. Doppler ultrasound was performed on fetuses prior to preterm labor/birth in dams injected with a monoclonal  $\alpha$ CD3 $\epsilon$  antibody (10 $\mu$ g dissolved in 200 $\mu$ L of sterile PBS; n=13) or time-matched isotype controls (10 $\mu$ g dissolved in 200 $\mu$ L of sterile PBS; n=12). Fetal heart rate (A) and umbilical artery pulsatility index (B) were recorded. Data are from 12-13 independent litters.

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