Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi: 10.1111/cei.13437

The effects of advanced maternal age on T-cell subsets at the maternal–fetal interface prior to term labor and in the offspring: a mouse study

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Accepted for publication 2 April 2020
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Summary

Women who conceive at 35 years of age or older, commonly known as advanced maternal age, have a higher risk of facing parturition complications and their children have an increased risk of developing diseases later in life. However, the immunological mechanisms underlying these pathological processes have yet to be established. To fill this gap in knowledge, using a murine model and immunophenotyping, we determined the effect of advanced maternal age on the main cellular branch of adaptive immunity, T cells, at the maternal–fetal interface and in the offspring. We report that advanced maternal age impaired the process of labor at term, inducing dystocia and delaying the timing of delivery. Advanced maternal age diminished the number of specific proinflammatory T-cell subsets [T helper type 1 (Th1): $CD4^+IFN\text{-}\gamma^+$, $CD8^+IFN\text{-}\gamma^+$ and Th9: $CD4^+IL\text{-}9^+$], as well as $CD4^+$ regulatory T cells ($CD4^+CD25^+FoxP3^+$ T cells), at the maternal–fetal interface prior to term labor. Advanced maternal age also altered fetal growth and survival of the offspring in early life. In addition, infants born to advanced-age mothers had alterations in the T-cell repertoire but not in $CD71^+$ erythroid cells ($CD3^-CD71^+TER119^+$ cells). This study provides insight into the immune alterations observed at the maternal–fetal interface of advanced-age mothers and their offspring.

Keywords: birth weight, neonate, offspring, pregnancy, preterm labor

Introduction

During the past three decades, the mean childbearing age has steadily increased in developed and high-income countries, due largely to social and career-based changes as well as advances in contraceptives and assisted reproductive technologies [1]. Women aged 35 years or older, commonly defined as being of advanced maternal age, now comprise a significant proportion of the pregnant population [1,2]. Such delayed pregnancy is associated with a wide range of perinatal complications, including a higher risk of developing hypertensive disorders [3-5] and gestational diabetes mellitus [6,7]. Additionally, women

of advanced maternal age more commonly face parturition complications such as dystocia/prolonged labor [8,9], indicated cesarean section [10-12] and maternal near-miss events or morbidity [13,14]. While the associations between advanced maternal age and these pregnancy complications are well established, the immunological mechanisms underlying these pathological processes, particularly prolonged labor, are poorly understood.

Labor is considered a state of systemic [15-19] and local [20-31] physiological inflammation [32-35]. The latter concept is supported by consistent evidence showing that labor is characterized by an increase in cellular and soluble inflammatory mediators in the cervix [32,36-48], myometrium [37-38,40,49-52], chorioamniotic membranes [38,40,53-59], and decidual tissues (i.e. maternal–fetal interface) [38,40,53-54,57,60-63]. Specifically, in the decidual tissues, the process of labor at term has been associated with proinflammatory phenotypes of macrophages (i.e. M1-like phenotype) [64] and effector T cells [65,66]. Indeed, these immune cell types are also detected in women who underwent the pathological process of preterm labor [64,65]. Therefore, we have proposed that a tight balance among the cellular components at the maternal–fetal interface is implicated in the physiological and pathological processes of labor [67-69]. However, whether alterations in the adaptive immune responses, specifically T cells, take place at the maternal–fetal interface in women of advanced maternal age has yet to be shown.

Beyond the pregnancy consequences associated with advanced maternal age, several studies reported that the children born to women of advanced maternal age have an increased risk of developing diseases later in life, including type 1 childhood diabetes [70-72], allergies [73], male infertility [74] and female menstrual disorders [75], among others [76-82]. While the etiology of some of these long-term sequelae remains unknown, most have no known linkage to maternal transmission of genes, defective mitochondria, or chromosomal abnormalities [83]. Furthermore, there is an increasing body of evidence suggesting that the intrauterine environment shapes developmental outcomes, including immunological development in the offspring [84-86]. This hypothesis, in tandem with the understanding that aging is characterized by chronic systemic inflammation [87] and that pregnancy is tightly regulated by the immune system [88-91], begs the question of how the intrauterine environment in women of advanced maternal age may alter T-cell responses in the offspring.

In the current study, we first evaluated the perinatal consequences of advanced maternal age using a murine model. Additionally, we performed immunophenotyping of decidual and splenic infantile murine T cells to determine the impact of advanced maternal age on the main cellular branch of adaptive immunity at the maternal–fetal interface and in the offspring, respectively.

Materials and methods

Mice

B6N.129(Cg)-forkhead box protein 3 (FoxP3)$^{tm3Ayr/J}$ mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA), bred in the animal care facility at the C. S. Mott Center for Human Growth and Development (Wayne State University, Detroit, MI, USA), and housed under a circadian cycle (light/dark = 12 : 12 h). This mouse strain was chosen because initial studies were focused on regulatory T cells (T_{regs}). Syngeneic mating was used to evaluate the effect of advanced maternal age as the sole variable. Older females [≥ 20 weeks, advanced maternal age (AMA)] were mated with males of proven fertility in three different cohorts: the first to obtain observational data and the second two for flow cytometry data. Young females (aged 8–12 weeks, ideal reproductive age, controls) were also mated with males of proven fertility as controls. The females were checked between 8:00 a.m. and 9:00 a.m. daily for the appearance of a vaginal plug indicating 0·5 days post-coitum (dpc), at which point female mice were removed from the mating cages and housed separately. Pregnancy was confirmed by a weight gain of ≥ 2 g at 12·5 dpc. All mouse experiments were approved by the Institutional Animal Care and Use Committee at Wayne State University (Protocol no. A-09-08-12). The authors adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Murine model of advanced maternal age

In the first cohort, fertility rates were recorded, defined as the proportion of mice who successfully became pregnant after the identification of a vaginal plug among the total number of mice with a vaginal plug. Pregnancy parameters, including duration of labor, rate of dystocia, and gestational length, were monitored via video camera (Sony, Tokyo, Japan). Duration of labor was defined as the time between delivery of the first and last pup in undisturbed, successful deliveries. The rate of dystocia was defined as the proportion of mice who underwent disturbed progression of labor (duration of labor > 6 h) among the total number of pregnant mice. Gestational length was calculated as the time from the presence of the vaginal plug until the observation of the first pup in the cage bedding. Litter sizes of all successful deliveries were recorded. After delivery, the mother and her pups were kept under observation, and offspring survival and weights were recorded 1, 2, and 3 weeks after birth.

Immunophenotyping by flow cytometry

Older and young dams from the second cohort were euthanized prior to term parturition, the day before delivery, on 18·5 dpc. The number of fetuses, fetal weights, and placental weights were recorded. Additionally, the maternal spleen, uterine draining lymph nodes (ULN), and decidual tissues were collected. In the third cohort, neonates (1 week) and infants (3 weeks) from older and young dams were weighed and euthanized, and spleens were collected. The isolation of leukocytes from lymphatic and decidual tissues was performed using mechanical dissociation followed by enzymatic disaggregation, as previously described [67,92]. The cells were incubated with anti-CD16/CD32 (FcγIII/II receptor; BD Biosciences, San Jose, CA, USA) for 10 min, followed by extracellular staining with specific fluorochrome-conjugated anti-mouse monoclonal antibodies (Supporting information, Table S1). For intracellular/intranuclear staining, the cells were first fixed and permeabilized using the Cytofix/Cytoperm fixation/permeabilization solution (Cat. no. 554714; BD Biosciences) or the FoxP3 staining buffer kit (Cat. no. 005523-00; eBiosciences, San Diego, CA, USA), respectively, prior to incubation with intracellular/intranuclear antibodies, which included staining for cytokines (Supporting information, Table S1). For the staining of $CD71^+$ erythroid cells, the 1X fluorescence activated cell sorter (FACS) lysing solution (BD Biosciences) was used. After staining, cell pellets were washed and resuspended in 0·5 ml FACS buffer. Samples were acquired using the BD LSRFortessa® flow cytometer (BD Biosciences) and analyzed with BD FACSDiva® Software version 7.0 (BD Biosciences). The analysis and figures were performed using FlowJo software version 10 (FlowJo, LLC, Ashland, OR, USA). The absolute number of cells was determined using CountBright absolute counting beads (Molecular Probes, Eugene, OR, USA).

T-cell phenotypes were determined in the maternal tissues and infantile tissues. Such immunophenotyping included the identification of: conventional T cells ($CD3^+$), $CD4^+$ T cells ($CD3^+CD4^+$), $CD8^+$ T cells ($CD3^+CD8^+$), T helper type 1 (Th1) cells [$CD3^+CD4^+IFN\text{-}\gamma^+$], Th2 cells [$CD3^+CD4^+IL\text{-}4^+$], Th9 cells ($CD3^+CD4^+IL\text{-}9^+$), Th17 cells ($CD3^+CD4^+IL\text{-}17A^+$), $CD8^+IFN\text{-}\gamma^+$ cells ($CD3^+CD8^+IFN\text{-}\gamma^+$), $CD8^+IL\text{-}4^+$ cells ($CD3^+CD8^+IL\text{-}4^+$), $CD8^+IL\text{-}9^+$ cells ($CD3^+CD8^+IL\text{-}9^+$), $CD8^+IL\text{-}17A^+$ cells ($CD3^+CD8^+IL\text{-}17A^+$), $CD4^+$ regulatory T cells ($CD3^+CD4^+CD25^+FoxP3^+$) and $CD3^+CD8^+CD25^+FoxP3^+$ cells. $CD71^+$ erythroid cells ($CD3^-CD71^+TER119^+$) were also identified in neonatal and infantile tissues and reported as proportions due to low cell counts.

Statistical analysis

Data were analyzed using SPSS Statistics software version 19.0 (IBM, Armonk, NY, USA). For the rates of fertility and dystocia, Fisher's exact test was used. Kaplan–Meier survival curves were used to plot and compare the gestational length and neonatal survival data (Mantel–Cox test). For the duration of labor, litter size, placental weights, offspring weights, and all flow cytometry data, the Shapiro–Wilk normality test was performed. For non-normally distributed data, the Mann–Whitney *U*-test was utilized to compare experimental data between the control and study groups. Alternatively, for normally distributed data, the unpaired *t*-test was performed. A *P*-value of $\leq 0{\cdot}05$ was considered statistically significant.

Results

The negative effects of advanced maternal age in pregnancy outcomes

Mice reach sexual maturity at ~6–8 weeks of age [93]; however, the mating age used in reproductive studies is between 8 and 12 weeks of age [65,94,95]. Therefore, in the current study, control mice were mated within this range (8–12 weeks of age), which represents the ideal reproductive age (Fig. 1a). In rodents, fertility begins to decline at approximately 6 months of age (~24 weeks), which mirrors the decline in fertility of women who are aged ~35–40 years [96]. Therefore, older females were allowed to reach 20–24 weeks of age, which represents the decline in fertility seen in women of advanced maternal age (AMA, Fig. 1a). Consistently, we found that AMA dams tended to have a lower fertility rate compared to controls [73.9% (17 of 23) *versus* 100% (13 of 13), Fig. 1b]. In addition, AMA dams that successfully reached term pregnancy had significantly longer durations of labor than controls (Fig. 1c), which is consistent with human data associating prolonged labor with increased maternal age [9]. Furthermore, the rate of dystocia was notably higher in the AMA group than the control group [35.2% (six of 17) *versus* 7·7% (one of 13), Fig. 1d]. The gestational length of AMA dams tended to be longer than that of controls (Fig. 1e). However, the litter size between AMA and control dams was not significantly different (Fig. 1f). These data show that advanced maternal age is associated with adverse effects during pregnancy, including impaired fertility, increased duration of labor and gestation and a higher frequency of dystocia.

Advanced maternal age alters the T-cell repertoire at the maternal–fetal interface

Previous studies have shown that T cells at the maternal–fetal interface participate in the physiological [57,65-66,69,97-99] and pathological [65,69,99-103] processes of labor. Therefore, we next quantified decidual T-cell subsets prior to term delivery (18·5 dpc) using flow

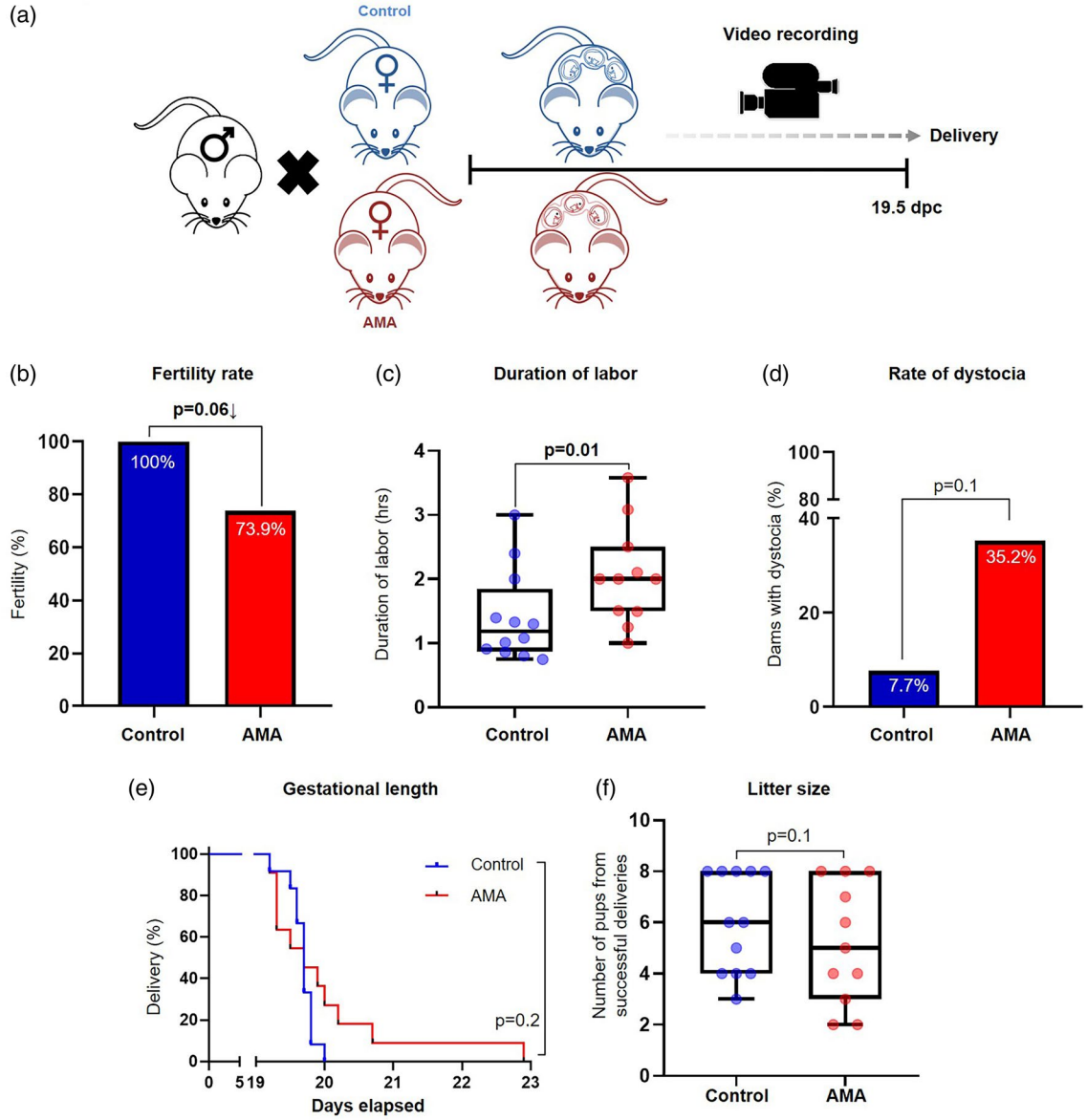

Fig. 1. Pregnancy outcomes of advanced maternal age. (a) Experimental design of advanced maternal age (AMA) during pregnancy. (b) Percentage of fertile control and AMA mice (n = 13–23 each). The P-values were determined by Fisher's exact test. (c) Duration of active labor in undisturbed, successful deliveries from control and AMA dams (n = 11–12 each) in control and AMA groups. Mid-lines indicate medians, boxes indicate interquartile ranges and whiskers indicate minimum–maximum range. The P-values were determined by a Mann–Whitney U-test. (d) Percentage of control and AMA dams who went into dystocia (n = 13–17 each). The P-values were determined by Fisher's exact test. (e) Kaplan–Meier survival curves showing the gestational lengths of control and AMA dams (n = 11–12 each). The P-values were determined by Mantel–Cox test. (f) Number of pups per litter from control and AMA dams who had successful deliveries (n = 11–12 litters). Mid-lines indicate medians, boxes indicate interquartile ranges and whiskers indicate minimum–maximum range. The P-values were determined by a Mann–Whitney U-test. Significant P-values are shown in bold type.

cytometry (Fig. 2a,b). We first determined the numbers of conventional T cells in the decidua, and found no differences in the decidual $CD4^+$ (Fig. 2c) or $CD8^+$ (Fig. 2d) T cells between AMA dams and controls. We next analyzed the expression of stereotypical cytokines associated with helper T-cell subsets: namely, IFN-γ (Th1) [14,105], IL-4 (Th2) [14,105], IL-9 (Th9) [16-109], and IL-17A (Th17) [110] (Fig. 2b, right panels). Advanced maternal age was associated with a marked reduction in decidual Th1 cells compared to controls (Fig. 2e).

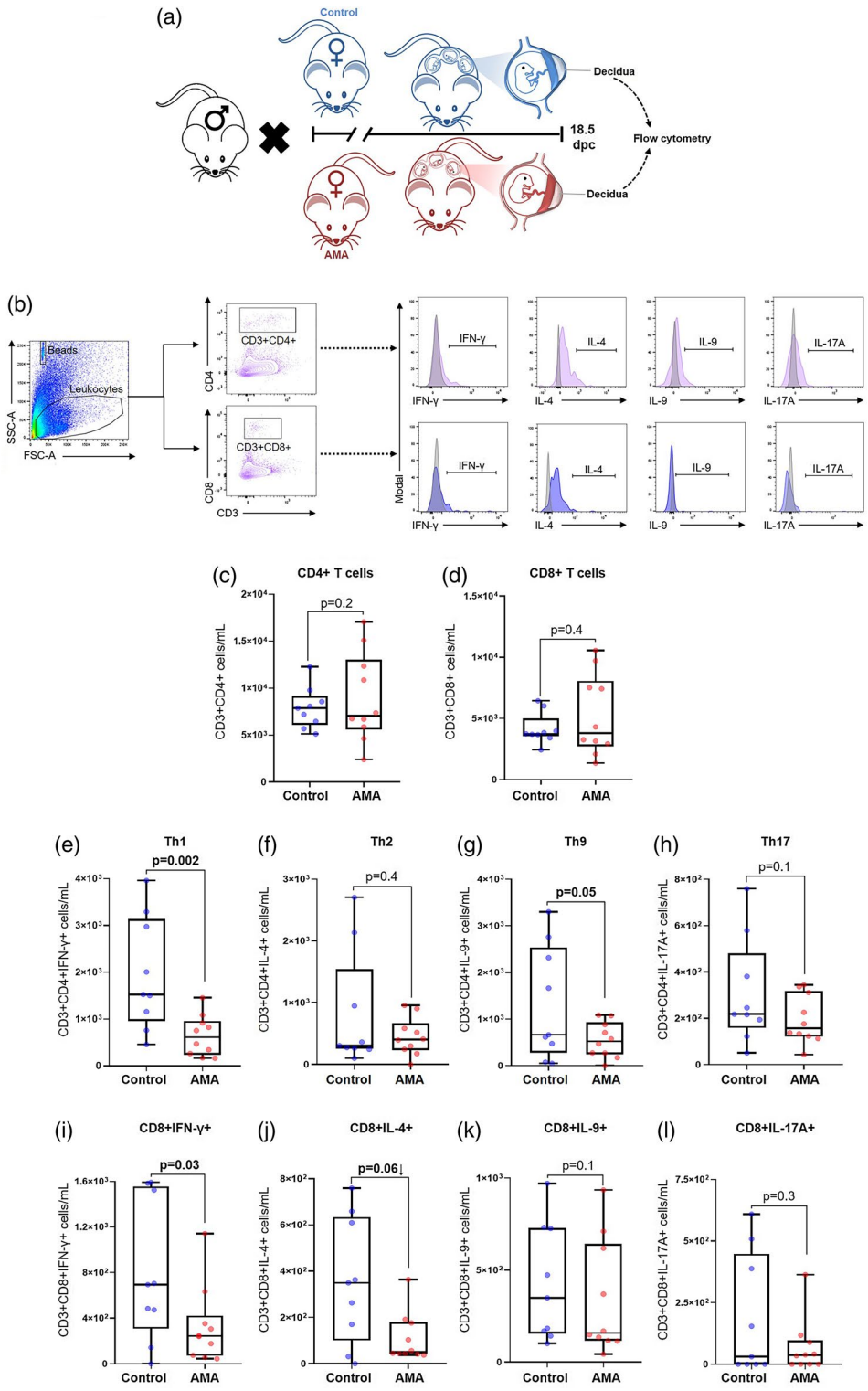

Fig. 2. Immunophenotyping of the T-cell subsets in the decidua of advanced maternal aged dams. (a) Experimental design of decidual tissue collection in murine model of advanced maternal age (AMA) and young controls. (b) Gating strategy used to quantify the T-cell subsets in the decidua. Number of (c) $CD4^+$ T cells, (d) $CD8^+$ T cells, (e) T helper type 1 (Th1) cells, (f) Th2 cells, (g) Th9 cells, (h) Th17 cells, (i) $CD8^+$ cells expressing IFN-γ, (j) $CD8^+$ cells expressing IL-4, (k) $CD8^+$ cells expressing IL-9, and (l) $CD8^+$ cells expressing IL-17A (n = 9–10 each). Mid-lines indicate medians, boxes indicate interquartile ranges and whiskers indicate minimum–maximum range. The P-values were determined by an unpaired t-test or a Mann–Whitney U-test. Significant P-values are shown in bold type.

Moreover, a modest but non-significant reduction was observed in the numbers of decidual Th9 cells in AMA dams compared to controls (Fig. 2g; $P = 0{\cdot}05$). Neither the number of decidual Th2 cells (Fig. 2f) nor the number of Th17 cells (Fig. 2h) were significantly altered by AMA. Among the corresponding $CD8^+$ T-cell subsets, there was a significant reduction in the population of $CD8^+IFN\text{-}\gamma^+$ T cells in AMA dams compared to controls (Fig. 2i), similar to the trend seen in the Th1 cells. There was also a modest decrease in $CD8^+IL\text{-}4^+$ T cells in AMA dams compared to controls (Fig. 2j; $P = 0{\cdot}06$). No differences were seen in the number of $CD8^+IL\text{-}9^+$ T cells (Fig. 2k) or $CD8^+IL\text{-}17A^+$ T cells (Fig. 2l) between the study groups. Notably, such alterations in T-cell populations were limited to the maternal–fetal interface, as no changes were observed in T-cell subsets in the maternal ULN or spleen between AMA dams and controls (Supporting information, Figs S1 and S2). Together, these results show that AMA impairs the proinflammatory T-cell responses at the maternal–fetal interface prior to parturition.

We and others have previously suggested that systemic and local T_{regs} play an important role in the timing of parturition [98,111-115]. Therefore, we quantified $CD4^+$ T_{regs} and $CD8^+CD25^+FoxP3^+$ T cells in the decidual tissues of AMA and control dams (Fig. 3a). We found that AMA dams had a significantly diminished population of decidual $CD4^+$ T_{regs} compared to controls (Fig. 3b). However, this reduction in $CD4^+$ T_{regs} was exclusively local, as no changes were observed in T_{regs} from the ULN or spleen of AMA and control dams (Supporting information, Fig. S3). By contrast, no differences were seen in the

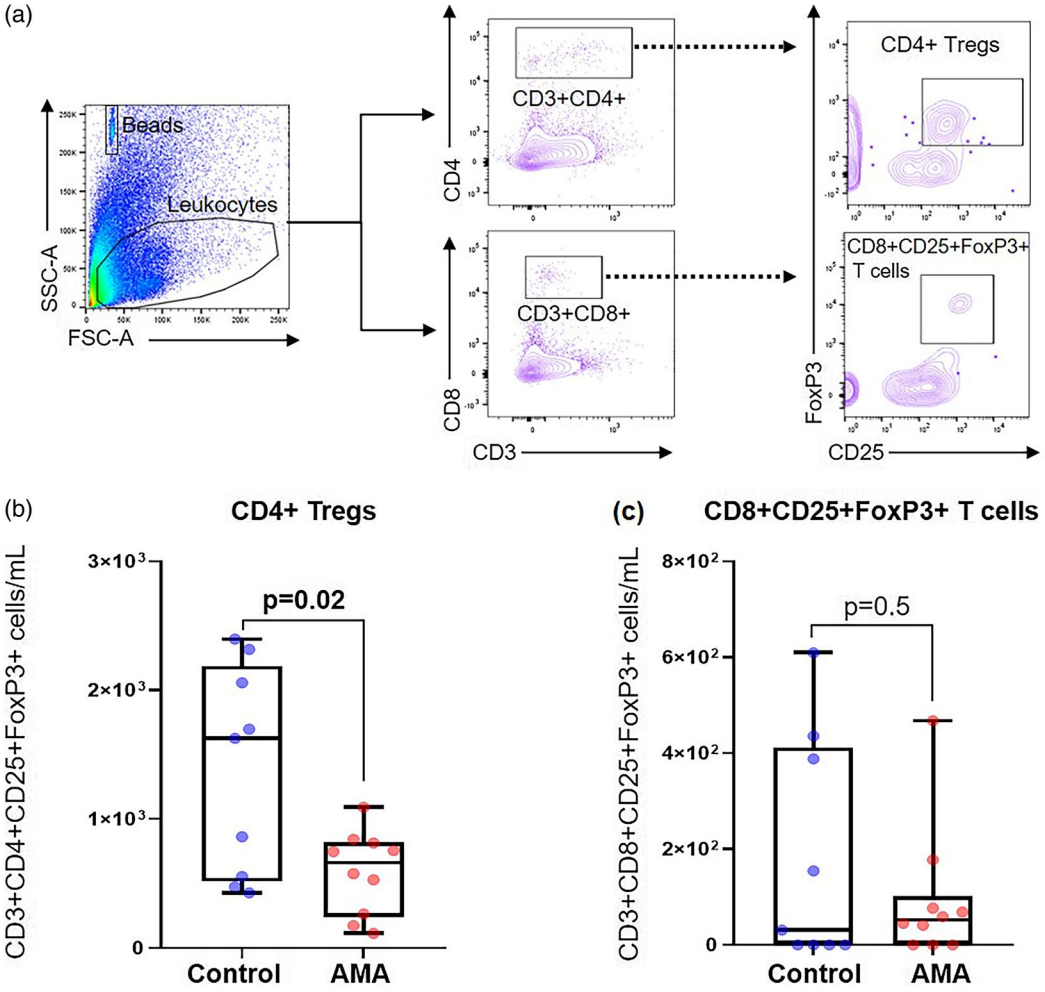

Fig. 3. Immunophenotyping of regulatory T cells in the decidua of advanced maternal aged (AMA) dams. (a) Gating strategy used to quantify the regulatory T cells (T_{regs}) in the decidua. Number of (b) $CD4^+$ T_{regs} and (c) $CD8^+CD25^+FoxP3^+$ T cells ($n = 9$–10 each) in control and AMA dams. Mid-lines indicate medians, boxes indicate interquartile ranges and whiskers indicate minimum–maximum range. The *P*-values were determined by an unpaired *t*-test or a Mann–Whitney *U*-test. Significant *P*-values are shown in bold type.

numbers of $CD8^+CD25^+FoxP3^+$ T cells between study groups (Fig. 3c). This finding demonstrates that AMA results in a reduction of $CD4^+$ T_{regs} at the maternal–fetal interface, but does not affect the $CD8^+CD25^+FoxP3^+$ T-cell population.

Advanced maternal age impairs neonatal survival and growth of the offspring

Given that AMA has been associated with adverse neonatal outcomes such as low birth weight [116] and a higher rate of stillbirth [117,118], we next investigated the impact of older maternal age on the immediate and long-term health of the offspring (Fig. 4a). There was a significant reduction in neonatal survival in the AMA group compared to controls, with neonatal mortality predominately occurring within the first week of life among the different litters (Fig. 4b). To further elucidate this discrepancy in neonatal survival and assess the long-term health of the offspring, placental and fetal weights at 18·5 dpc as well as neonatal growth trajectories were compared between AMA mice and controls. There was no difference in the placental weights at 18·5 dpc between the study groups (Fig. 4c). However, there was a significant reduction in the fetal weights from AMA dams at 18·5 dpc compared to controls (Fig. 4d), indicating that the pup-to-placenta weight ratio contributes to AMA-related neonatal consequences. Interestingly, neonates born to AMA dams were significantly heavier than those from young controls at 1 week of age (Fig. 4e). This disparity in offspring growth was overcome by 3 weeks of age, as a difference was not observed between the infants from AMA dams and controls (Fig. 4f). These results provide supporting evidence showing that AMA not only impairs the process of labor, but also affects the early survival and growth of the offspring.

Advanced maternal age disrupts T-cell phenotypes in infants

Infants born to AMA dams were apparently healthy; however, there is a substantial body of literature associating advanced maternal age with multiple disorders in the offspring later in life [70-83]. Therefore, we evaluated T-cell responses in infants born to AMA dams to determine the integrity of the cellular limb of their adaptive immune system. First, we characterized the conventional T-cell repertoire in the infant spleen (Fig. 5a,b). Infants from AMA dams had modestly increased numbers of splenic $CD4^+$ T cells compared to those from controls (Fig. 5c; $P = 0{\cdot}05$). Moreover, the numbers of splenic $CD8^+$ T cells were also elevated in infants from AMA dams compared to those from controls (Fig. 5d). We next investigated splenic helper T-cell subsets in the offspring of AMA dams, and found that the numbers of Th1 cells were significantly increased compared to the offspring of controls (Fig. 5e). Similarly, splenic Th2 T cells were also augmented in infants from AMA dams (Fig. 5f; $P = 0{\cdot}05$). However, the numbers of Th9 cells (Fig. 5g) and Th17 cells (Fig. 5h) remained stable when compared between the study groups.

In line with these findings, the numbers of splenic $CD8^+IFN\text{-}\gamma^+$ T cells were significantly elevated in the offspring of AMA dams compared to controls (Fig. 5i), as were the numbers of splenic $CD8^+IL\text{-}4^+$ T cells (Fig. 5j). There were no differences in the numbers of splenic $CD8^+IL\text{-}9^+$ T cells (Fig. 5k) or in the numbers of splenic $CD8^+IL\text{-}17A^+$ T cells (Fig. 5l) between infants from AMA dams and those from controls.

We also characterized splenic $CD4^+$ T_{regs} from the infants of AMA and control dams (Fig. 6a). Consistent with the numbers of conventional T cells, the numbers of splenic $CD4^+$ T_{reg} cells were increased in infants from AMA dams compared to those from controls (Fig. 6b; $P = 0{\cdot}05$). Moreover, the numbers of splenic $CD8^+CD25^+FoxP3^+$ T cells were also elevated in infants from AMA dams compared to those from controls (Fig. 6c). These results suggest that the offspring from advanced-age mothers who survive past the neonatal window undergo compensatory alterations in the T-cell repertoire.

Advanced maternal age does not alter infant CD71+ erythroid cells

There is an increasing body of evidence reporting that neonatal innate and adaptive immunity depends on the critical immunomodulatory functions of $CD71^+$ nucleated erythroid cells [119-124]. Indeed, we have shown that $CD71^+$ erythroid cells play a central role by modulating immune responses in neonates born to mothers who underwent the process of preterm or term labor [125,126]. Therefore, we investigated the effect of AMA on the $CD71^+$ erythroid cell population of the offspring at 1 and 3 weeks of age (Fig. 7a). The proportions of $CD71^+$ erythroid cells in the offspring of AMA dams were unaffected at 1 week of age compared to those from controls (Fig. 7b). Similarly, $CD71^+$ erythroid cells were unchanged at 3 weeks after birth (Fig. 7c). These findings show that AMA does not affect the proportion of $CD71^+$ erythroid cells in the offspring; however, further studies are required to investigate whether AMA alters the functionality of these cells.

Discussion

The current study provides evidence that advanced maternal age: (1) impairs fertility, the process of labor, and the timing of delivery; (2) diminishes the number of specific proinflammatory T-cell subsets (Th1, Th9, and $CD8^+IFN\text{-}\gamma^+$) at the maternal–fetal interface prior to term parturition;

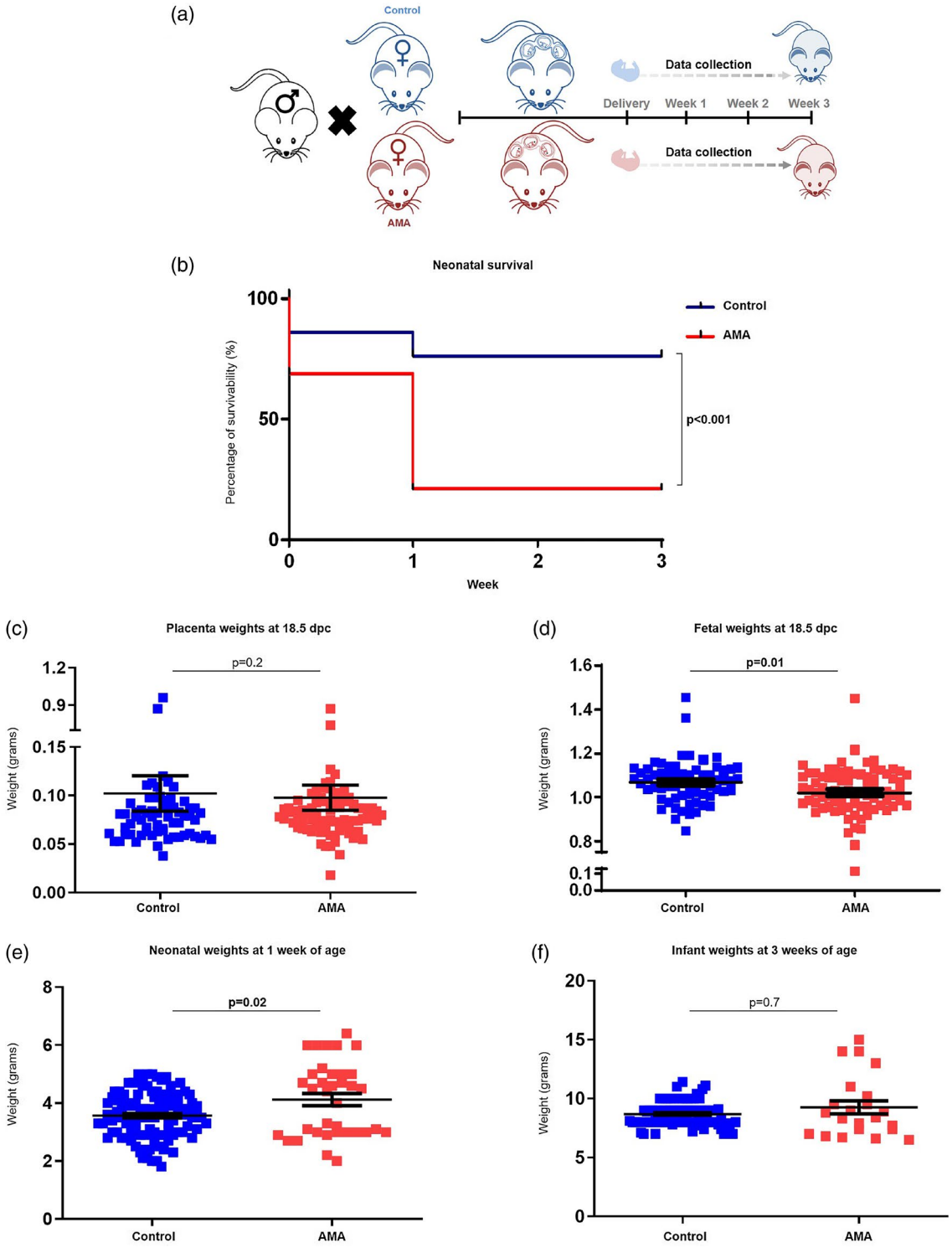

Fig. 4. Neonatal outcomes in advanced maternal aged dams. (a) Experimental design of data collection from the offspring of advanced maternal aged (AMA) and control dams. (b) Kaplan–Meier survival curves showing the rate of survival of offspring from AMA dams and controls at birth and 1, 2, and 3 weeks of life. The *P*-values were determined by Mantel–Cox test. (c) Weights of the placentas from controls and AMA dams at 18·5 days post-coitum (dpc). Weights of the offspring from control and AMA dams at (d) 18·5 dpc, (e) 1 week and (f) 3 weeks of age (n = 22–103 each). Data are shown as scatter-plots mean with standard error of the mean (s.e.m.)]. The *P*-values were determined by a Mann–Whitney *U*-test. Significant *P*-values are shown in bold type.

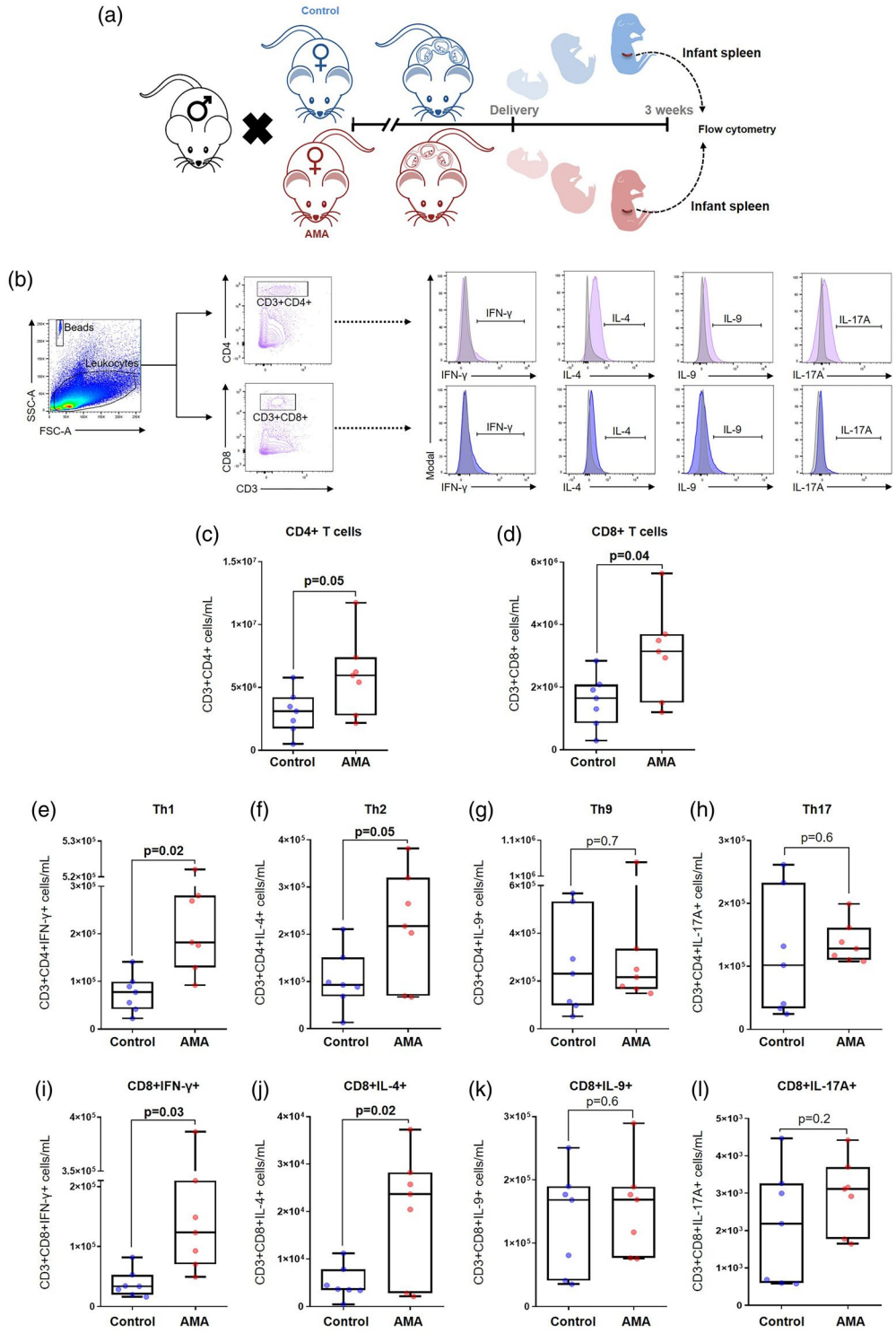

Fig. 5. Immunophenotyping of T-cell subsets in the spleen of infants from advanced maternal aged dams. (a) Experimental design of infant spleen collection from offspring of control and advanced maternal aged (AMA) dams. (b) Gating strategy used to quantify the T-cell subsets in the infant spleen. Number of (c) $CD4^+$ T cells, (d) $CD8^+$ T cells, (e) T helper type 1 (Th1) cells, (f) Th2 cells, (g) Th9 cells, (h) Th17 cells, (i) $CD8^+$ cells expressing IFN-γ, (j) $CD8^+$ cells expressing IL-4, (k) $CD8^+$ cells expressing IL-9, and (l) $CD8^+$ cells expressing IL-17A ($n = 7$ each). Mid-lines indicate medians, boxes indicate interquartile ranges and whiskers indicate minimum–maximum range. The *P*-values were determined by an unpaired *t*-test or a Mann–Whitney *U*-test. Significant *P*-values are shown in bold type.

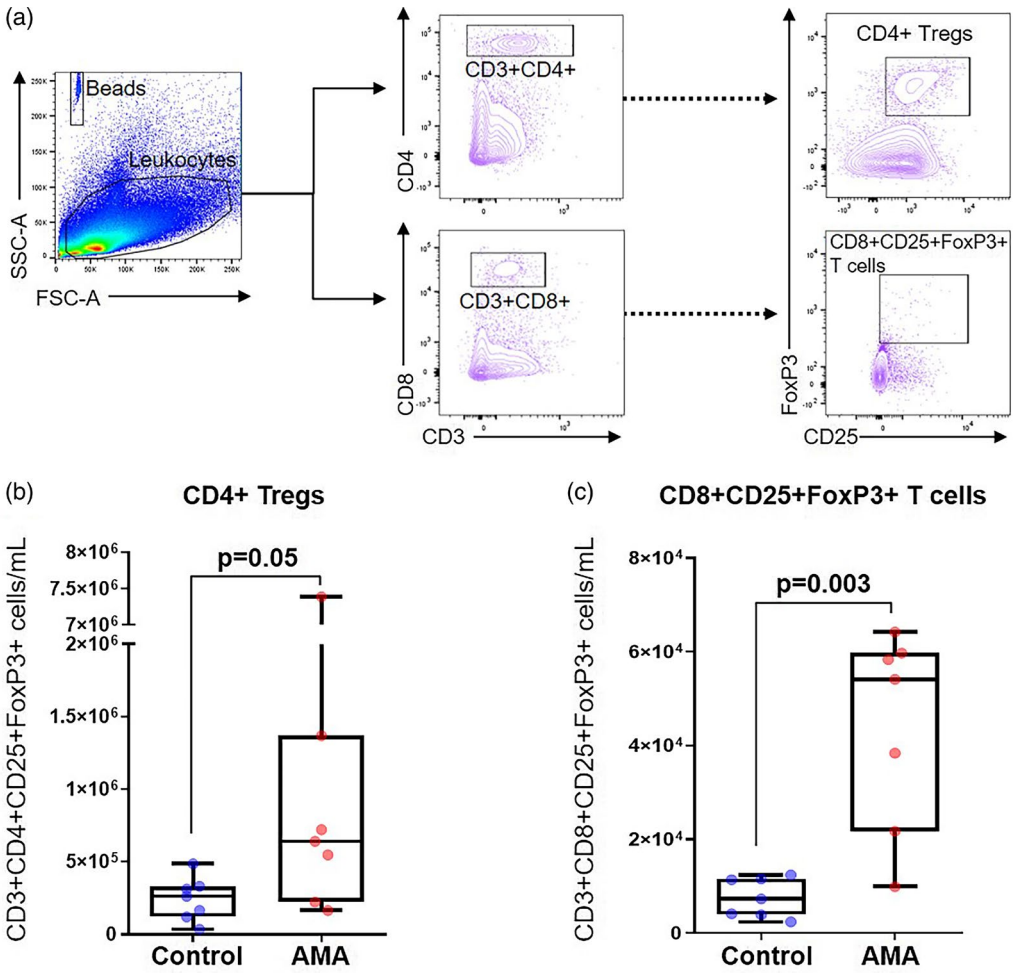

Fig. 6. Immunophenotyping of regulatory T cells (T_{regs}) in the spleen of infants from advanced maternal aged (AMA) dams. (a) Gating strategy used to quantify the T_{regs} in the infant spleen. Number of (b) $CD4^+$ T_{regs} and (c) $CD8^+CD25^+FoxP3^+$ T cells ($n = 7$ each) in control and AMA dams. Mid-lines indicate medians, boxes indicate interquartile ranges and whiskers indicate minimum–maximum range. The *P*-values were determined by an unpaired *t*-test or a Mann–Whitney *U*-test. Significant *P*-values are shown in bold type.

(3) reduces the number of $CD4^+$ T_{regs}, but not $CD8^+CD25^+FoxP3^+$ T cells, at the maternal–fetal interface prior to parturition; (4) alters the growth and survival of the offspring in early life; (5) induces an expansion of IFN-γ- and IL-4-producing $CD4^+$ and $CD8^+$ T cells, as well as $CD4^+$ T_{regs} and $CD8^+CD25^+FoxP3^+$ T cells, in the infant; and (6) does not alter the proportion of $CD71^+$ erythroid cells in the offspring. Collectively, these findings provide a phenotypical characterization of the effects of advanced maternal age on T-cell responses at the maternal–fetal interface prior to term labor and in the offspring.

In recent years, a growing body of evidence suggests a role for maternal T cells in the processes of labor: (1) T cells are actively recruited from the periphery into the decidual tissues through chemotactic processes during the onset of term labor [57,97,127]; (2) T cells, including exhausted T cells, are enriched at the human maternal–fetal interface prior to the onset of term labor [99,128-135]; (3) T-cell exhaustion at the maternal–fetal interface is reversed by inflammatory mediators associated with term labor [99]; (4) activated T cells at the maternal–fetal interface express labor mediators such as tumor necrosis factor (TNF)-α, IL-1β, and matrix metalloproteinase (MMP)-9 during the process of term labor [66]; (5) effector T cells expressing perforin are increased at the maternal–fetal interface of women with term labor compared to non-labor controls [65]; (6) IL-6, a cytokine that participates in the timing of parturition [136], controls decidual T-cell subsets prior to term labor [98]; (7) activation of T cells by administration of an anti-CD3 antibody induces the process of preterm labor [13]; (8) effector and activated T cells expressing granzyme B and perforin are enriched at the maternal–fetal interface of women who underwent spontaneous preterm labor and

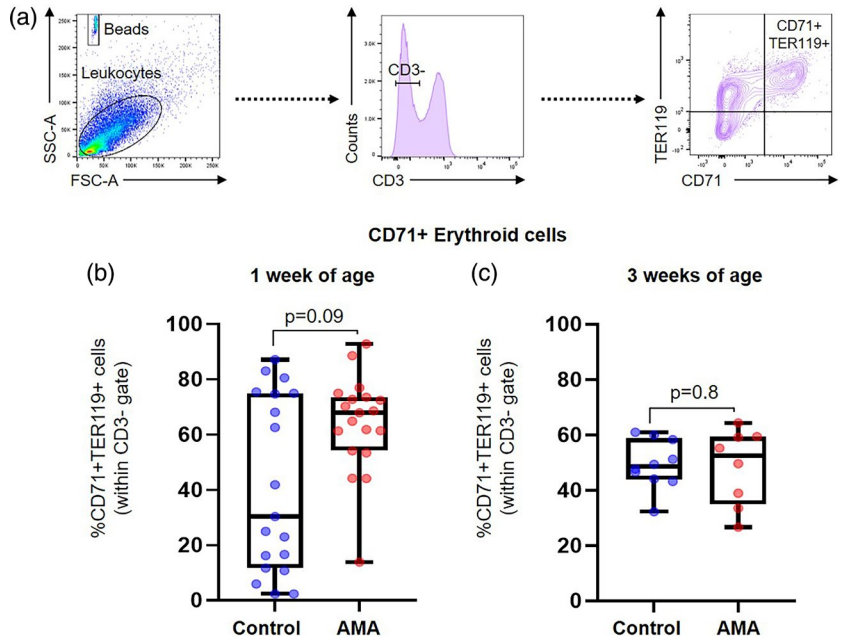

Fig. 7. Immunophenotyping of CD71$^+$ erythroid cells in the spleen of offspring from advanced maternal aged (AMA) dams. (a) Gating strategy used to quantify CD71$^+$ erythroid cells. Proportion of CD71$^+$ erythroid cells (b) in the neonatal spleen at 1 week of age and (c) in the infant spleen at 3 weeks of age (n = 8–19 each) in control and AMA groups. Mid-lines indicate medians, boxes indicate interquartile ranges and whiskers indicate minimum–maximum range. The P-values were determined by an unpaired t-test or a Mann–Whitney U-test. Significant P-values are shown in bold type.

birth [65]; and (9) single-cell RNA signatures of activated T cells precede term and preterm labor [69,90]. Herein, we put forward evidence that advanced maternal age is associated with a pathological delay of the process of term labor, and this is associated with alterations in T-cell subsets at the maternal–fetal interface.

The mechanisms whereby advanced maternal age induces dystocia and extended duration of labor may involve alterations in the proinflammatory milieu localized at the maternal–fetal interface. For example, we found that mothers of advanced age had fewer Th1 and Th9 cells in the decidual tissues. These T-cell subsets have been previously reported at the maternal–fetal interface [98,137,138]. The differentiation of Th1 and Th9 cells results from stimulation with specific cytokines such as IL-12/IFN-γ [139-141] and IL-4/transforming growth factor (TGF)-β1 [18-109,142], respectively. Therefore, it is tempting to suggest that the decidual tissues of advanced-age mothers are deficient in local cytokines required for the differentiation of the Th1 and Th9 subsets. Herein, we also report that advanced-age mothers had fewer IFN-γ- and IL-4-expressing CD8$^+$ T cells at the maternal–fetal interface. This observation may also reflect defective inflammatory signaling pathways in the decidual tissues of advanced-age mothers. Further investigation is required to elucidate the molecular mechanisms whereby advanced maternal age causes impaired T-cell subset differentiation at the maternal–fetal interface prior to term labor.

CD4$^+$ T_{regs} are an important subset of T cells that express CD25 and FoxP3 [143-146]. These cells play a central role in immune tolerance by exhibiting suppressive activity towards both self- and non-self-antigens [147-149]. This suppressive activity is due largely to the expression of the transcription factor FoxP3 [144,150]. CD4$^+$ T_{regs} have been localized at the human [151,152] and murine [67,153,154] maternal–fetal interface. These cells seem to play an important role in promoting maternal–fetal tolerance from early to mid-pregnancy [153,155-157]. However, this T-cell subset has not been involved in the timing of parturition at term, as shown herein at the maternal–fetal interface of advanced-age mothers. The cellular mechanisms (e.g. TGF-β1 signaling) whereby advanced age causes a reduction in the number of CD4$^+$ T_{regs} in the decidua are worthy of further research.

Our study provides a phenotypical characterization of the T-cell subsets that are defective at the maternal–fetal interface of advanced-age mothers. However, it is important to note that aging of the reproductive organs [158-162], as well as other pathological processes [163-166], must be considered when studying the mechanisms whereby advanced maternal age causes prolonged labor. It is worth mentioning that the pathological processes involved in

prolonged labor are confined to the intrauterine space, given that the alterations in T-cell subsets were not observed in the lymphatic tissues.

In this study, we also report that advanced maternal age is associated with reduced early offspring survival, which is in tandem with our finding of reduced fetal weight. This is consistent with prior studies that have associated advanced maternal age in mice with decreased offspring body weight and life expectancy [167-169]. Future studies are warranted to determine whether this finding is due to postnatal factors such as maternal caring behaviors or differences in breast milk quantity and composition, or antenatal and perinatal factors that occur *in utero*. We also found that advanced maternal age alters T-cell subsets, including $CD4^+$ T_{regs}, without affecting other immunomodulatory cells (e.g. $CD71^+$ erythroid cells) in infants. To our knowledge, this is the first demonstration that infants of mothers of advanced age have increased numbers of $CD4^+$ and $CD8^+$ T cells expressing IFN-γ, IL-4, and FoxP3. This rise may be due to a compensatory mechanism in response to the adverse/extended intrauterine environment [84,85], which is reflected by the appropriate weight gain in infants at 3 weeks of age. A similar compensatory mechanism has also been observed in infants born to mothers who experienced chronic prenatal stress [170]. Nevertheless, the immunocompetence of pups born to dams of advanced age remains to be elucidated.

Conclusion

The data in the current study provide evidence that advanced maternal age impairs the process of labor and alters the T-cell repertoire at the maternal–fetal interface prior to term labor. Additionally, we show that advanced maternal age is associated with adverse consequences for the offspring, as demonstrated by affected growth patterns and T-cell responses. Together, these findings represent the first characterization of the effect of advanced maternal age on the main cellular branch of adaptive immunity, T cells, at the maternal–fetal interface prior to term labor and in the offspring. These findings provide insight into the immune mechanisms dysregulated in the pathological process of delayed labor and, more importantly, suggest that infants born to mothers of advanced age may display impaired T-cell immunity.

Acknowledgements

This research was supported by the Wayne State University Perinatal Initiative in Maternal, Perinatal and Child Health and the Perinatology Research Branch, Division of Obstetrics and Maternal–Fetal Medicine, Division of Intramural Research, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, US Department of Health and Human Services (NICHD/NIH/DHHS); and, in part, with federal funds from NICHD/NIH/DHHS under Contract no. HHSN275201300006C. R. R. has contributed to this work as part of his official duties as an employee of the United States Federal Government.

Disclosures

The authors have no financial conflicts of interest.

Author contributions

D. L., V. G-F., D. M. and Y. X. performed research and analyzed and interpreted data. R. R. interpreted data and provided guidance in the experimental design. A. S. analyzed and interpreted data. S. S. H. provided intellectual input. N. G.-L. designed research, interpreted data, and provided supervision throughout the study. All authors participated in the writing of the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Fig. S1. Immunophenotyping of the T-cell subsets in the uterine draining lymph nodes (ULN) of advanced maternal aged (AMA) dams. Number of (a) CD4+ T cells, (b) CD8+ T cells, (c) Th1 cells, (d) Th2 cells, (e) Th9 cells, (f) Th17 cells, (g) CD8+ cells expressing IFNγ, (h) CD8+ cells expressing IL-4, (i) CD8+ cells expressing IL-9, and (j) CD8+ cells expressing IL-17A (n = 6-10 each) in control and AMA dams. Mid-lines indicate medians, boxes indicate interquartile ranges, and whiskers indicate min-max range. The p values were determined by an unpaired t test or a Mann-Whitney U test.

Fig. S2. Immunophenotyping of the T-cell subsets in the spleen of advanced maternal aged (AMA) dams. Number of (a) CD4+ T cells, (b) CD8+ T cells, (c) Th1 cells, (d) Th2 cells, (e) Th9 cells, (f) Th17 cells, (g) CD8+ cells expressing IFNγ, (h) CD8+ cells expressing IL-4, (i) CD8+ cells expressing IL-9, and (j) CD8+ cells expressing IL-17A (n = 7-10 each) in control and AMA dams. Mid-lines indicate medians, boxes indicate interquartile ranges, and whiskers indicate min-max range. The p values were determined by an unpaired t test or a Mann-Whitney U test.

Fig. S3. Immunophenotyping of regulatory T cells (Tregs) in the uterine draining lymph nodes (ULN) and spleen of advanced maternal aged (AMA) and control dams. Number of (a) CD4+ Tregs and (b) CD8+CD25+FoxP3+ T cells in the ULN (n = 6-10 each). Number of (c) CD4+ Tregs and (d) CD8+CD25+FoxP3+ T cells in the spleen (n = 7-10 each). Mid-lines indicate medians, boxes indicate interquartile ranges, and whiskers indicate min-max range. The p values were determined by an unpaired t test or a Mann-Whitney U test.

Table S1. Antibodies used for immunophenotyping.