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Elevated maternal retinoic acid-related orphan receptor-γt enhances the effect of polyinosinic-polycytidylic acid in inducing fetal loss

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Abstract: T helper 17 (Th17) cells have been suggested to play a crucial role in various complications during pregnancy by participating in maternal immune activation (MIA). To test a possible role for Th17 cells in MIA-mediated abortion, we analyzed transgenic mice overexpressing retinoic acid receptor-related orphan receptor gamma-t (RORγt), a master regulator of IL-17 producing cell development. These mutant mice (RORγt Tg mice) exhibited a constitutive upregulation of serum IL-17A and decreased E-cadherin expression in cell–cell junctions of placental tissues. Abortion after the administration of a viral-mimicking synthetic double-stranded RNA polyinosinic–polycytidylic acid was more frequent in RORγt Tg mice than wild-type mice. These results suggest that excessive Th17 cell activity alters immune responsiveness and increases the rate of abortion during gestation.

Key words: abortion, IL-17, maternal immune activation, polyinosinic-polycytidylic acid [poly(I:C)], retinoic acid-related orphan receptor gamma-t (RORγt)

Introduction

The maintenance of pregnancy is susceptible to inflammation, which is activated by various viral/bacterial infections and immune disorders. However, the mechanisms underlying pregnancy loss because of harmful immune responses remain elusive.

T helper 17 (Th17) cells, a lineage of $CD4^+$ T helper cells, have been shown to be important in the host defense against infectious agents [3]. Previous studies also suggest that Th17 cells participate in pregnancy pathogenesis such as spontaneous abortions [5]. However, the details of abortion associated with Th17 are poorly understood. Th17 cells are constitutively present in the lamina propria of the gut, and stimulation by IL-6 and transforming growth factor-β increases their population [18]. Activation of the transcription factor retinoic acid receptor-related orphan receptor gamma-t (RORγt) is required for Th17 cell differentiation [9].

IL-17 (IL-17A and IL-17F) is a cytokine that is released from Th17 cells and elicits broad proinflammatory responses from various tissues and cell types known

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to express the IL-17 receptor complex [18]. IL-17 levels were reported to be higher in the placenta and peripheral blood of pregnant women with recurrent pregnancy loss (PRL) compared with healthy pregnant women [14, 23]. Moreover, the administration of recombinant IL-17 to pregnant mice increased the abortion rate [24], while the rate of lipopolysaccharide-induced fetal loss in murine pregnancies correlates well with higher levels of IL-17 [13]. These reports indicate the importance of IL-17 in abortions.

In the present study, we analyzed transgenic mice expressing excessive RORγt under the control of the T-cell specific CD2 promoter (RORγt Tg mice: [15, 26]) to examine the role of IL-17-producing T cells in complications during pregnancy. RORγt is a crucial transcriptional regulator for the development of Th17 cells, as well as IL-17 producing $CD8^+$ T cells (Tc17), invariant natural killer T (iNKT) cells and γδT cells[8, 9, 16]. We showed that RORγt Tg mice exhibited higher rates of fetal loss than wild-type (WT) mice when maternal immune activation (MIA) was triggered by polyinosinic-polycytidylic acid [poly(I:C)], a viral mimicking potent inducer of inflammation that causes fetal loss in pregnant female mice [22]. Surprisingly, the observed fetal loss was not accompanied by poly(I:C)-induced increases of IL-17A.

Materials and Methods

Animals

C57BL/6J mice were obtained from Japan CLEA (Tokyo, Japan). RORγt Tg mice generated on a C57BL/6J background in which transgene expression was driven by the CD2 promoter [26] were obtained from Takahashi Laboratory (University of Tsukuba, Tsukuba, Japan). The RORγt Tg mouse line was maintained by backcrossing with C57BL/6J mice. All animals were housed under standard SPF laboratory conditions (12/12 h light/dark cycle, with free access to food and water). All experiments were carried out according to the Guide for the Care and Use of Laboratory Animals at the University of Tsukuba, and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). All efforts were made to minimize animal suffering and the number of animals used.

Quantitative real-time PCR

Small intestine samples for real-time PCR experiments were obtained from male WT and RORγt Tg mice at 10 weeks of age. Total RNA was extracted from mouse intestines using NucleoSpin RNA (Macherey-Nagel, Düren, Germany), and cDNA was synthesized using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Real-time PCR was performed on an Eco Real-time PCR System (Illumina, San Diego, CA, USA) with a THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan). Analyses were performed in duplicate. Relative expression levels were calculated with the $2^{-\Delta\Delta CT}$ method using HPRT as an internal control. Primers were as follows: RORγt forward, 5'-TGAGGCCATTCAGTATGTGG-3'; RORγt reverse, 5'-CTTCCATTGCTCCTGCTTTC-3'; HPRT forward, 5'-TTGTTGTTGGATATGCCCTTGACTA-3'; and HPRT reverse, 5'-AGGCAGATGGCCACAGGACTA-3'.

Abortion rate

Timed pregnancies were generated by housing pairs of females with a single male overnight. Animals were separated the next day, and 12:00 p.m. on this day was classified as embryonic day (E) 0.5 for these studies. On E12.5, pregnant female mice were weighed and injected with a single dose (20 mg/kg; intraperitoneally, i.p.) of poly(I:C) (Sigma-Aldrich, St. Louis, MO, USA) or phosphate-buffered saline (PBS; vehicle) and sacrificed at 48 h after the injection (Supplementary Fig. 1). Seven pregnant mice were sacrificed for each group. The uteri were removed, and implantation sites were documented. Abortion sites were identified by their small size accompanied by a necrotic, hemorrhagic appearance compared with healthy embryos and placentas (Supplementary Fig. 1).

ELISA

Blood samples (>0.5 ml) for ELISA were obtained from the hearts of non-pregnant (10 weeks of age) and pregnant (10–18 weeks of age, first pregnancy) mice at 2, 6, 24, and 48 h after the poly(I:C) injection (20 mg/kg; i.p. at E12.5). Four mice per group (WT and RORγt Tg) were used for this analysis. Blood was collected in tubes containing EDTA-2K (Capiject II, Terumo, Tokyo, Japan) and centrifuged at 3,000 g at 4°C for 10 min. Note that 0 h group mice received no injections and were sacrificed on E12.5. Serum concentrations of IL-17A

were measured using the ELISA system (PM 1700, R&D Systems, Minneapolis, MN) following the manufacturer's instructions.

Immunohistochemistry

For histological analysis, pregnant mice were deeply anesthetized with sodium pentobarbital (60 mg/kg body weight (BW), i.p. injection; Somnopentyl, BCM International, Hillsborough, NJ). The placentas were collected from non-treated pregnant WT and RORγt Tg mice on E12.5 and fixed overnight with 4% paraformaldehyde at 4°C, followed by rinsing three times with PBS. The placentas were then immersed in 30% sucrose in PBS until the tissue sank for cryoprotection at 4°C. The tissues were embedded in OCT compound (Sakura Finetek Japan, Tokyo, Japan), and snap-frozen in nitrogen. Frozen tissue was stored at −80°C until further use. Placental sections (10 μm) were prepared and mounted onto MAS-coated glass slides (Matsunami Glass, Kishiwada, Japan), permeabilized with 0.3% Triton X-100 in PBS for 30 min at room temperature, blocked with 10% normal goat serum in PBS for 1 h at room temperature, followed by incubation with anti-E-cadherin antibody (1:100, #ab11512, Abcam, Cambridge, UK) overnight at 4°C. After washing three times with PBS, they were then incubated with Alexa Fluor 568-conjugated goat anti-rat IgG secondary antibody (1:500, #A11077, Invitrogen, Carlsbad, CA, USA) in PBS for 1 h at room temperature, washed three times with 0.05% Tween-20 in PBS, treated with 4',6-diamidino-2-phenylindole (DAPI; 1:1000, Thermo Fisher Scientific, Waltham, MA, USA) in PBS to visualize the nuclei, washed three times with PBS, and mounted with a cover glass (Matsunami Glass) using PermaFluor Aqueous Mounting Medium (Thermo Fisher Scientific).

Image analysis

Fluorescent images were acquired on an LSM 510 confocal laser microscope (Carl Zeiss, Oberkochen, Germany) with a 20× objective lens, and analyzed with ImageJ software (NIH). E-cadherin expression was calculated as the integrated density (integrated area × mean gray value) of E-cadherin / integrated density of DAPI nuclear staining.

Statistical analysis

Differences between the two groups were analyzed with the Student's *t*-test. Fisher's exact test was applied for comparisons of abortion rates of different groups (Fig. 2 and Supplementary Table 1). Two-way analysis of variance (ANOVA) and Shaffer's modified sequentially rejective Bonferroni procedure post-hoc test was used to analyze IL-17A serum concentrations (Fig. 3) and BW of non-pregnant mice (Supplementary Fig. 2). All statistical analyses were performed using Python. Probability values <0.1 were considered marginally significant and probability values <0.05 were considered significant. All data are expressed as means ± SEM.

Results

Overexpression of RORγt mRNA and increased synthesis of IL-17A in RORγt Tg mice

Th17 cells are typically abundant in the mouse small intestinal mucosa [25]. To confirm *RORγt* overexpression in RORγt Tg mice, we examined *RORγt* mRNA expression in the small intestine of WT and RORγt Tg mice by quantitative real-time PCR. *RORγt* mRNA was expressed at more than twice the level in RORγt Tg mice as in WT mice (Fig. 1A, Student's *t*-test, $P<0.05$).

To investigate whether the amount of IL-17 released into the blood was increased by overexpressing *RORγt*, we measured serum IL-17A concentrations using ELISA and found that it was significantly elevated in RORγt Tg compared with WT mice (Fig. 1B, 2.72 ± 1.11 pg/ml versus 9.54 ± 1.77, respectively, Student's *t*-test, $P<0.05$). These results indicate that excessive polarization into Th17 cells and constitutive upregulation of IL-17A occurred in RORγt Tg mice.

The poly(I:C)-induced abortion rate was higher in RORγt Tg than in WT mice

To determine the role of RORγt in MIA-associated abortion, we used a poly(I:C) model of MIA because poly(I:C) was previously shown to induce significant fetal loss [22]. We compared abortion rates between WT and RORγt Tg mice treated with PBS or poly(I:C), respectively, by identifying abortion sites as small, necrotic, and hemorrhagic uteri in pregnant mice (Supplementary Fig. 1). In the group administered with PBS, there was no significant difference in abortion rates between WT (3.7%) and RORγt Tg (7.3%) mice (Control, Fisher's exact test, $P=0.679$). However, a significant decrease in the number of surviving fetuses was observed in both WT and RORγt Tg pregnant mice after poly(I:C) treatment (Fig. 2 and Supplementary Table 1; 52 to 36

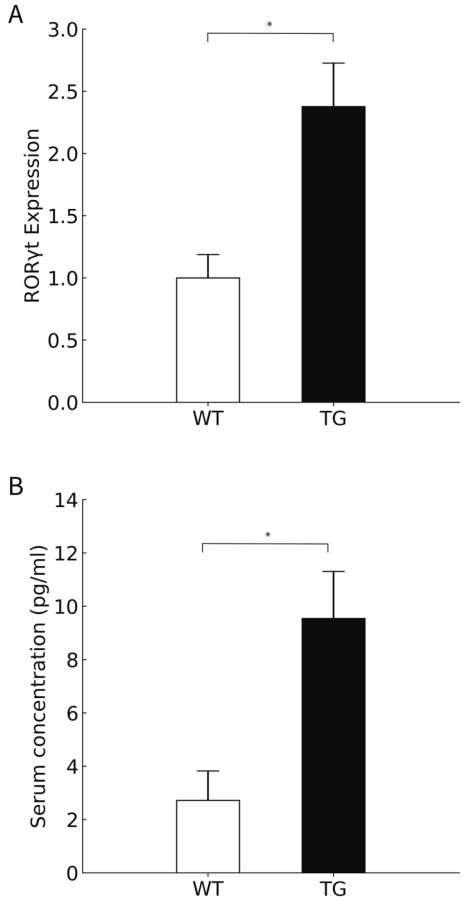

Fig. 1. Intestinal *RORγt* mRNA and serum IL-17A expression. (A) *RORγt* mRNA expression was significantly higher in the small intestine of RORγt Tg mice than in that of wild-type (WT) mice at 10 weeks of age. n=4 per group, Student's *t*-test **P*<0.05. (B) Serum IL-17A concentrations were significantly higher in RORγt Tg than in WT mice. n=4 per group, Student's *t*-test **P*<0.05.

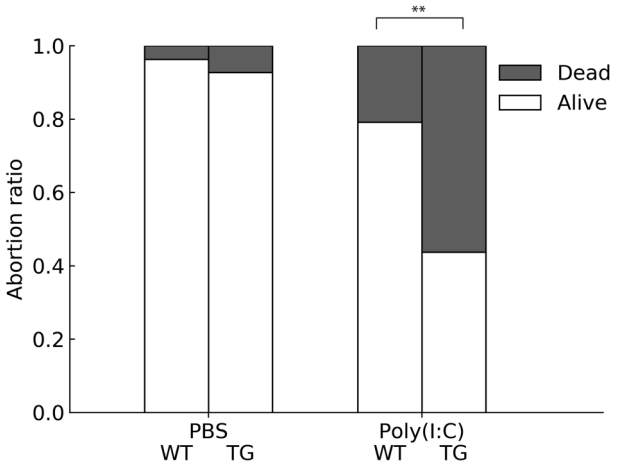

Fig. 2. Fetal loss at 48 h after maternal phosphate-buffered saline (PBS) or polyinosinic-polycytidylic acid [poly(I:C)] administration. Pregnant mice were intraperitoneally injected with PBS or poly(I:C) (20 mg/kg) at E12.5. Poly(I:C) administration induced more fetal loss in retinoic acid receptor-related orphan receptor gamma-t (RORγt) Tg mice than in wild-type (WT) mice. n=7 per group, Fisher's exact test ***P*<0.01 (WT 0.971 ± 0.0535, RORγt 0.929 ± 0.0695 in PBS; WT 0.814 ± 0.113, RORγt Tg 0.417 ± 0.175 in poly(I:C); mean ± SEM).

in WT following poly(I:C) treatment, and 51 to 21 in RORγt Tg after poly(I:C) treatment). Importantly, the percentage of fetal loss after poly(I:C) administration was significantly higher in RORγt Tg (56.3%) than in WT (21.7%) mice (Fig. 2 and Supplementary Table 1; n=7, Fisher's exact test, *P*<0.01). Typical hemorrhagic areas were more often observed in the uteri of poly(I:C)-challenged mice than in WT mice (Supplementary Fig. 1), suggesting that excessive IL-17 producing T cells exacerbate the poly(I:C)-induced pregnancy loss.

Time course of IL-17A level after poly(I:C) treatment

To gain insights into the contribution of IL-17 to poly(I:C)-induced abortions, we compared the level of IL-17A after poly(I:C) treatment between RORγt Tg and WT mice. Blood samples were collected from non-pregnant and pregnant mice at 0, 2, 6, 24, and 48 h after poly(I:C) injection, and the serum concentration of IL-17A was analyzed by ELISA.

In non-pregnant mice, IL-17A concentrations were consistently upregulated in RORγt Tg compared with WT mice (Fig. 3A; two-way ANOVA, effect of genotype: F (1, 30)=36.15, *P*<0.01). Serum IL-17A concentrations were not significantly increased by poly(I:C) administration in both WT and RORγt Tg mice in the non-pregnant state (Fig. 3A). To confirm the impact of poly(I:C) administration, the BW of non-pregnant WT and RORγt Tg mice was monitored every 24 h after poly(I:C) injection (Supplementary Fig. 2). A temporal decrease of BW was similarly observed in both WT and RORγt Tg mice (two-way ANOVA; effect of sampling time: F (2, 12)=39.01, *P*<0.01). There was no difference in the time-course of BW between genotypes, indicating that poly(I:C) administration induced similar reactions in both WT and RORγt Tg mice.

The time course of IL-17A after poly(I:C) injection differed between pregnant and non-pregnant mice, and also between genotypes (two-way ANOVA; effect of sampling time: F (4, 30)=12.65, *P*<0.01; genotype × sampling time interaction F (4; 30)=14.54, *P*<0.01). In

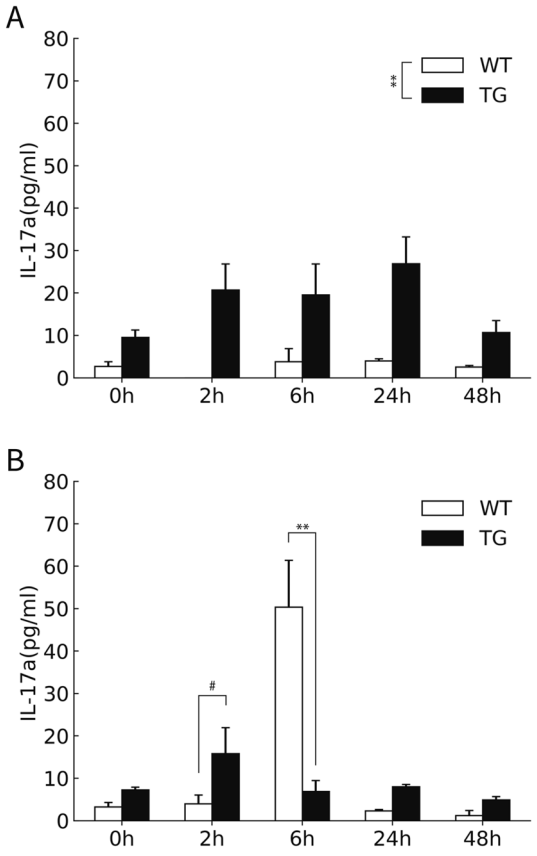

Fig. 3. Polyinosinic-polycytidylic acid [poly(I:C)]-induced change of serum IL-17A levels. Data of (A) non-pregnant (10 weeks of age) and (B) pregnant mice (10–16 weeks of age) are shown. IL-17A was measured at 2, 4, 6, 24, and 48 h after 20 mg/kg poly(I:C) administration.

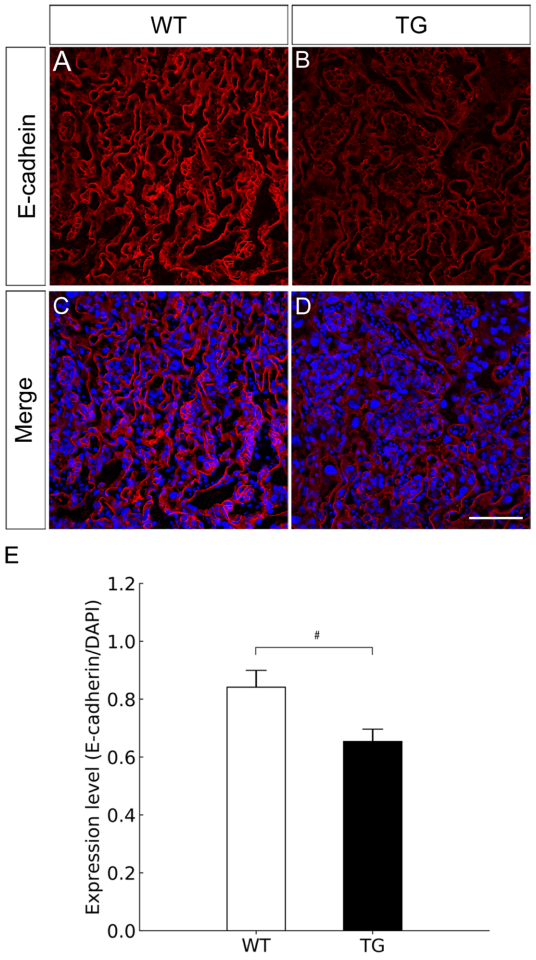

Fig. 4. Expression of E-cadherin in the placenta. (A and B) Distribution pattern of E-cadherin in the labyrinth layer of the placenta in wild-type (WT) (A) and retinoic acid receptor-related orphan receptor gamma-t (RORγt) Tg (B) mice. (C and D) DAPI nuclear staining (blue) was merged with E-cadherin staining (red). Scale bar=100 μm. (E) Relative fluorescent intensity of the E-cadherin immunohistochemical signal was compared between RORγt Tg and WT mice at E12.5. Three mice per group were examined. Student's *t*-test #P<0.1. E-cadherin expression was significantly lower in RORγt Tg than in WT mice, indicative of loosening of adherens junctions in the placenta of RORγt Tg mice. Six sections per animal were analyzed for quantification.

WT mice, IL-17A showed significant increases after poly(I:C) treatment, with highest levels observed 6 h later when the concentration was around 10 times higher than at 0 h, after which it decreased to baseline levels (<5.0 pg/ml, Fig. 3B). Unexpectedly, RORγt Tg mice showed no increase in serum IL-17A concentrations after poly(I:C) injection (Fig. 3B). These results suggest that the prominent increase of poly(I:C)-induced fetal loss in RORγt Tg mice is not mediated by the acute upregulation of IL-17A, but by other poly(I:C)-related immune reactions.

Disrupted integrity of adherens junctions in the placenta of RORγt Tg mice

The marked increase in abortion rate induced by poly(I:C) administration in RORγt Tg mice suggested the possibility of placental vulnerability. To test this, we examined the cellular structure of the placenta by immunohistochemistry using an antibody against E-cadherin, which is expressed in the labyrinth layer of the placenta [20] and contributes to the formation of adherens junction [1, 19]. In WT mice, E-cadherin signals delineated a fine, close-knit branched structure at E12.5 (Fig. 4A). However, the signal was faint in RORγt Tg mice (Fig. 4B). E-cadherin signal quantification revealed weaker signal intensities in RORγt Tg mice compared

with WT (Fig. 4E, 0.84 ± 0.058 versus 0.65 ± 0.043, respectively; Student's *t*-test, t=2.59, P<0.1). No difference was observed in cell densities between the two groups (depicted by DAPI-positive nuclei; Figs. 4C and D), suggesting that cell–cell adhesion of the labyrinth layer of the placenta is weakened in RORγt Tg mice.

Discussion

MIA is considered to be one of the major causes of abortion [6, 17], but little is known about the immunological details underlying MIA-induced abortions. In the present study, we found that overexpression of RORγt significantly enhanced fetal loss caused by poly(I:C)-induced MIA. We also observed reduced expression of membrane E-cadherin in the labyrinth zone of the placenta of RORγt Tg mice compared with WT mice. A mouse model of preterm labor generated by treatment with poly(I:C) showed a decrease in placental level of E-cadherin expression, possibly due to a loosening of adherens junctions caused by inflammatory destruction [10]. Moreover, serum levels of IL-17A, the major cytokine released from Th17 cells [18], were consistently upregulated in RORγt Tg mice, suggesting that the alterations in E-cadherin expression were caused by IL-17-mediated chronic immune responses.

In spite of the changes in their placental tissue adherens junctions, RORγt Tg mice maintained their pregnancies at comparable levels to WT mice (Fig. 2). However, the administration of poly(I:C) led to significantly higher incidences of abortion in RORγt Tg mice than in WT mice (Fig. 2). Because IL-17A is strongly implicated in recurrent pregnancy loss and other pregnancy-related pathological statuses in humans [5], we examined the time-course of its expression after poly(I:C) administration. In WT mice, serum IL-17A was significantly upregulated in response to poly(I:C) injection only in pregnant mice (Figs. 3A and B), which is consistent with previously reported findings [2, 11]. The mechanism of the elevated responsiveness of IL-17A production in pregnant mice remained unknown. Alternation of gut bacteria in pregnant state [12], which regulate maternal immune response, may relate with the dependence of IL-17A upregulation on pregnancy. Contrary to our expectations, RORγt overexpression did not enhance the poly(I:C)-induced upregulation of serum IL-17A, but instead suppressed its response after poly(I:C) injection (Fig. 3B). These paradoxical reactions prompted us to consider that Th17/IL-17 maintained at constitutively high levels in RORγt Tg mice might activate inhibitory immune systems to suppress IL-17 production [7].

Multiple regulatory pathways are known to inhibit IL-17, including Th1, Th2, and regulatory T (Treg) cells, and interleukins such as IL-4 and INF-γ [4]. Among these regulatory systems, the balance between Th17 and Treg cells is reported to be especially important for the maintenance of pregnancy [4, 21]. We consider that a continuous excess of Th17 cells caused by RORγt overexpression might potentiate these inhibitory systems to suppress the increase of IL-17A after poly(I:C) administration, and that cytokines and interferons other than IL-17, such as IL-10, could be responsible for the poly(I:C)-induced abortion in RORγt Tg mice [22].

In summary, our data indicate that poly(I:C) induces IL-17-independent inflammatory responses in RORγt Tg mice, which act synergistically with the disruption of placental tissue caused by chronic upregulation of IL-17, leading to an increased rate of abortion. Our data also suggest that the rate of abortion associated with viral infection could be strongly affected by Th17 cell function. Additional research is needed to further elucidate details about the pathological effect of Th17 in MIA-induced abortion.

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