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Depletion of CD8⁺ Cells Abolishes the Pregnancy Protective Effect of Progesterone Substitution with Dydrogesterone in Mice by Altering the Th1/Th2 Cytokine Profile

Sandra M. Blois,*† Ricarda Joachim,* Judith Kandil,* Ricardo Margni,† Mareike Tometten,* Burghard F. Klapp,* and Petra C. Arck²

One of the most remarkable immunological regulations is the maternal immune tolerance toward the fetal semiallograft during pregnancy, which has been referred to as immunity’s pregnant pause. Rejection of the semiallogeneic trophoblast cells must be selectively inhibited and pathways presumably include Th2 cytokines unopposed by Th1 cytokines. Steroid hormones, including progesterone, have similar effects. Low levels of progesterone and Th2 cytokines and high levels of Th1 cytokines are attributable for increased abortions in mammalian species, which may be triggered by psychoemotional stress. Thus, the aim of the present study was to provide experimental evidence for the mechanism involved in the mediation of immune responses by endocrine signals during pregnancy and stress-triggered pregnancy failure. DBA/2J-mated CBA/J female mice were randomized in three groups: 1) control females, 2) mice exposed to stress on gestation day 5.5, and 3) mice exposed to stress and substituted with dydrogesterone, a progestogen with a binding profile highly selective for the progesterone receptor on gestation day 5.5. On gestation days 7, 9.5, and 10.5, mice of each group were sacrificed, and the frequency of CD8⁺ cells and cytokine expression (IL-4, IL-12, TNF-α, IFN-γ) in blood and uterus cells was evaluated by flow cytometry. Additionally, some mice were depleted of CD8 cells by injection of mAb. We observed that progesterone substitution abrogated the abortogenic effects of stress exposure by decreasing the frequency of abortogenic cytokines. This pathway was exceedingly CD8-dependent, because depletion of CD8 led to a termination of the pregnancy protective effect of progesterone substitution.


Often, the embryo has been referred to as “the mating product of histoincompatible individuals,” and consequently—was compared with a semiallogeneic graft having to be tolerated by the maternal immune system over the full gestational period (1).

The spatial adjacencies between fetal and maternal tissues guarantee nourishment of the fetus and the placenta, which is composed of fetal and maternal structures and an area for intense exchanges for nutrients and oxygen (1). In contrast, due to the presence of paternal Ags expressed by the placenta, rejection via the maternal immune system has to be avoided (2–5). Published data indicate that—during pregnancy—the maternal immune system appears to “be aware” of the fetal Ags, thus, tolerance mechanisms have evolved during evolution to ensure the maintenance of the fetoplacental graft (6).

However, rejection of the fetus and subsequent miscarriages are a frequent pregnancy complication affecting 15–40% of human pregnancies (7, 8). Inevitably, the fetoplacental graft may thus either be tolerated or rejected by the maternal immune system. The pathways of fetal tolerance appear to be plural and matter at diverse times in gestation. Intriguingly, these tolerance mechanisms act systemically on the maternal immune system and locally at the placental level. Indeed, the decision of survival or rejection of the fetus is particularly dependent on the events endangering the mother, prevalently environmental parameters such as exposure to toxic substances, infectious agents, or psychoemotional stress (9).

Systemic effects of fetal tolerance are first and foremost mediated by immunoactive hormones, e.g., progesterone, the hormone of pregnancy. In humans, progesterone-receptor antagonists promptly induce abortion if given before 7 wk of gestation (10). Likewise, surgical removal of the source of progesterone, the corpus luteum, results in pregnancy loss in rodents (11–14), suggesting that adequate progesterone production is critical to the maintenance of pregnancy. Interestingly, psychoemotional stress is known to generally inhibit the female reproductive system primarily through inhibition of progesterone (15, 16).

Locally at the fetomaternal interface, numerous mechanisms are involved and prevalently intertwined, e.g., expression of HMC class I is reduced on the syncytiotrophoblast, and the specific expression of HLA-G and HLA-E may block the cytolytic activity of NK cells (17, 18). Further, indoleamine 2, 3 dioxygenase, which catalyzes tryptophan, can be detected at the fetomaternal interface and has recently been reported to famish the local maternal immune response by depriving the T cells of tryptophan and/or by inhibiting lymphocyte proliferation (19, 20).

Recent evidence, much of it emerging from research on animal models, indicates important roles for T cells and cytokines in causing pregnancy failure (4, 21–24). It has been proposed that an inflammatory response mediated by macrophages, NK cells, and T cells with increased levels of Th1 cytokines may mediate abortions (25, 26). Based on the observations of deleterious effects of

*Charité, Department of Internal Medicine, Biomedizinisches Forschungszentrum, Humboldt University of Berlin, Berlin, Germany; and ¹Humoral Immunity Studies Institute-National Council of Scientific and Technological Research, University of Buenos Aires, Buenos Aires, Argentina

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² Address correspondence and reprint requests to Dr. Petra C. Arck, Biomedizinisches Forschungszentrum, Raum 2.0549, Augustenburger Platz 1, 13353 Berlin, Germany. E-mail address: petra.ark@charite.de

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Th1-type cytokines on mammalian pregnancy and on observations that pregnancy appears to be associated with a down-regulation of cell-mediated immunity manifested as reduced delayed-type hypersensitivity, it has been suggested that the Th1/Th2 balance could be a critical and a complex factor in the tolerance of the conceptus (8).

Data have indicated that a shift of Th1 to Th2 cytokines and suppression of NK cell cytolytic activity may be under the control of an immunomodulatory protein known as progesterone-induced blocking factor (PIBF) (27). PIBF is secreted by T cells, predominantly γδ TCR+ and CD8+ (24), upon interaction of progesterone with the progesterone receptors on such cells (28).

In successful pregnancies after immunotherapy (using the male partner’s blood as the source of leukocytes), systemic CD8+ progesterone-receptor+ cells were found to be significantly increased compared with preimmunotherapy (29). Because much of our understanding of early human pregnancy is inferred from studies in animals, we recently performed a pilot study to investigate the effect of the progesterone derivative dydrogesterone (6-dehydroprogesterone), a progesterone with a binding profile highly selective for the progesterone receptor (30), in stress-triggered murine abortion (31) and observed that stressed animals present lower systemic levels of progesterone and PIBF and a reduced expression of progesterone receptor at the fetomaternal interface (30).

Application of stress

The abortion rate was calculated as follows: % abortion rate = A/(A + V) × 100, whereby A means the number of dead placental units and V means viable placental units. The bars depict the mean percentage ± SEM; *, represents p < 0.05, as analyzed by the nonparametric Mann-Whitney U test.

Materials and Methods

Animals

Mice were purchased from Charles River Breeding Laboratories (Sulzfeld, Germany) and maintained in an animal facility with a 12 h light/dark cycle. Animal care and experimental procedures were followed according to institutional guidelines and conformed to the requirements of the state authority for animal research conduct (LaGetSi, Berlin, Germany). DBA/2J- and CBA/J-mated CBA/J female mice were randomized in different groups of females: 1) control mice that received s.c. injection of sterile filtered sesame oil (200 µl) on gestation day 5.5. The mice were sacrificed either on gestation day 7.5 (n = 7), 9.5 (n = 7), 10.5 (n = 7), or 13.5 (n = 7) for evaluation of the abortion rate and number of implants; 2) mice that were exposed to sound stress on gestation day 5.5 and also received a s.c. injection of 200 µl of sterile filtered sesame oil. Again, the mice were sacrificed either on gestation day 7.5 (n = 7), 9.5 (n = 7), 10.5 (n = 7), or 13.5 (n = 7) for evaluation of the abortion rate and number of implants; 3) mice that were exposed to gestation day 5.5 and received a s.c. injection of progesterone (provided by Solvay Pharmaceutical, Hannover, Germany) at a concentration of 1.25 mg/200 µl of sterile filtered sesame oil on gestation day 5.5, before exposure to sound stress was commenced. As in 1) and 2), the mice were sacrificed either on gestation day 7.5 (n = 7), 9.5 (n = 7), 10.5 (n = 7), and 13.5 (n = 7) for evaluation of the abortion rate and number of implants; 4) in a second experiment, an additional group of mice (n = 20) was treated as in 3), and divided into two subgroups (n = 10 each) which received s.c. injection of anti-Lyt 2.1 (cat. no. 558733) (85 µg/200 ml PBS) CD8 depletion on day 6.5 or PBS alone. Because we knew from the previous experiments on the mice described in 1-3) that gestation day 7.5 was a crucial day with respect to changes of the ureter cytokine equilibrium, we exclusively focused on the presence of cytokines on this particular day in the CD8 depletion experiments in five mice per subgroup in addition to the evaluation of the abortion rate and number of implants on day 13.5 (n = 5 for each subgroup).

FIGURE 1. Stress exposure increases abortion in mice, which is abrogated by dydrogesterone treatment via CD8-dependent pathways. A. Effect of stress exposure on gestation day 5.5 and dydrogesterone application during pregnancy on abortion rate, as determined on day 13.5 of gestation. The abortion rate was calculated as follows: % abortion rate = A/(A + V) × 100, whereby A means the number of dead placental units and V means viable placental units. The bars depict the mean percentage ± SEM; *, represents p < 0.05, as analyzed by the nonparametric Mann-Whitney U test. B. Effect of stress exposure and dydrogesterone application during pregnancy on the abortion rate in CD8-depleted mice.

FIGURE 2. Stress exposure, dydrogesterone treatment, or CD8 depletion did not affect number of implantations. Effect of stress exposure, dydrogesterone application, and CD8 depletion during pregnancy on the number of implantations on the various experimental gestational days. This figure depicts the total number of implantations, which comprise viable placental units (pointed out by the gray arrows in the photo of a uterus taken on gestation day 13.5) in addition to hemorrhagic units as an indicator for dead placental units (white arrows), thus the photo depicts an abortion rate of 50%. The data in the diagram are depicted as the mean ± SEM; no significant differences between the groups and days, respectively, were analyzed by the nonparametric Mann-Whitney U test.

3 Abbreviations used in this paper: PIBF, progesterone-induced blocking factor; RT, room temperature.
The number of dead placental units and cells were purified by Lympholyte/M (cat. no. GTS5030XK; Cedarlane was repeated twice, with HBSS medium containing no DTT. Mononuclear lymphocyte population in stressed, dydrogesterone-treated mice with and without DCD depletion on gestation day 7.5.

Abortion rate

Mice were sacrificed by neck dislocation on day 13.5, the uteri were removed, and the total number of implantations and resorbing sites (signs of abortion) was recorded. The abortion rate was calculated as percentages of means viable placental units.

Blood cells

On gestation days 7.5, 9.5, and 10.5, mice of the respective groups were narcotized and blood cells were obtained by retro-orbital puncture and washed twice with sterile PBS. Blood cells were used for determination of phenotype (surface expression) and cytokine expression on cells by flow cytometry.

Uterus cell isolation

Uteri were removed and uterus cells were isolated by the method described by Marquez et al. (32), with some modifications. In brief, the uterus was washed with sterile PBS, carefully cut into small pieces, and collected in tubes containing heparin. After treatment with ammonium chloride lysis buffer for 10 min to deplete erythrocytes, the cells were washed twice with sterile PBS. Blood cells were used for determination of phenotype (surface expression) and cytokine expression on cells by flow cytometry.

Flow cytometric analysis

For flow cytometry, the blood and uterus cells were incubated for 3 h with brefeldin A (10 μM/ml medium with 1 μg of Golgi Plug, 55-2301KZ; BD PharMingen, Heidelberg, Germany) in RPMI 1640 with FCS in a humidified incubator at 37°C with 5% CO₂. Flow cytometry was performed using our standard protocol (31): briefly, uterus cells were washed twice with FACS buffer (PBS supplemented with 1% BSA (cat. no. A-9418; Sigma-Aldrich) and 0.1% sodium acide (Sigma-Aldrich)). Cells were then incubated for 30 min at room temperature (RT) with FITC-labeled Ab against CD8α (cat. no. 553030). After the cells were washed and fixed using Fix solution (BD Biosciences, Erembodegem, Belgium), they were incubated for 30 min at RT in the dark. Subsequently, the cells were washed and permeabilized, using FACS Permeabilizing Solution (BD Biosciences), followed by incubation with intracellular Ab PE-labeled TNF-α (cat. no. 554419), IL-4 (cat. no. 554435), IFN-γ (cat. no. 554412), IL-12 (cat. no. 554479), and IL-10 (cat. no. 554467) for 30 min at RT in the dark. As a control, cells were stained with the corresponding isotype-matched mAb. All mAbs were purchased from BD Biosciences. The cells were then washed and read. The acquisition was performed using a FACS Calibur (BD Biosciences). Instrument compensation was set in each experiment using single-color stained samples. Data were analyzed by using CellQuest software. Flow cytometry results were expressed as the percentage of cells positive for the surface marker evaluated.

Statistical analysis

Statistical significance was determined using the nonparametric Mann-Whitney U test. Significance was set at p < 0.05.

Results

Stress-triggered increase of the abortion rate could be abrogated by the progestosterone derivative dydrogesterone

As depicted in Fig. 1A, we reproduced our previous findings that exposure to stress on gestation day 5.5 significantly boosts the abortion rate in DBA/2J-mated CBA/J females, as analyzed on gestation day 13.5, when resorption = abortion sites are visible (31). Further, substitution of progesterone with 1.25 mg of dydrogesterone on gestation day 5.5 significantly abrogated the stress-triggered increase of abortions. Neither stress nor dydrogesterone

**Figure 3.** CD8-positive decidual lymphocytes are decreased after stress exposure, dydrogesterone treatment restores them. A, The percentage of CD8⁺ cells within the uterine lymphocyte population in the various experimental groups on gestation days 7.5, 9.5, and 10.5. The data were obtained by flow cytometry and are presented as mean ± SEM. *, p < 0.05, **, p < 0.01, ***, p < 0.001, as analyzed by the nonparametric Mann-Whitney U test. B, The percentage of CD8⁺ cells within the uterine lymphocyte population in stressed, dydrogesterone-treated mice with and without CD8 depletion on gestation day 7.5.

**Figure 4.** Stress exposure up-regulates the percentage of TNF-α-positive decidual cells; dydrogesterone abolishes this proinflammatory response. Percentage of TNF-α-positive cells within the uterine lymphocyte population of the various experimental groups on gestation days 7.5, 9.5, and 10.5. TNF-α-positive cells were identified by flow cytometry for intracellular proteins. The data are presented as mean ± SEM, *, p < 0.05, **, p < 0.01, as evaluated by nonparametric Mann-Whitney U test.
On gestation day 10.5, 5 days after stress exposure, levels of CD8+ cells had been restored back to levels seen in control mice. Interestingly, on day 9.5, when the percentages in controls significantly decreased compared with day 7.5, this Th1 down-regulation was not present in stressed mice. When the stressed mice were treated with dydrogesterone, IFN-γ down-regulation did occur in uterine cells. On day 10.5, significant differences with respect to IFN-γ+ uterine cells were no longer detectable as the relative number was rather low compared with day 7.5. The absolute numbers of CD8+/IFN-γ+ and IL-12+ uterine cells are presented in Table II.

When looking at IL-12 expression in uterine cells, we observed no significant differences between the three groups and the different days of gestation (Fig. 6).

As presented in Fig. 7, stressed mice treated with dydrogesterone showed significantly higher percentages of pregnancy protective IL-4+ uterine cells beginning on gestation day 7.5 and, even more strikingly, maintained until day 10.5. The absolute numbers of CD8+/IL-4+ uterine cells are presented in Table II.

Depletion of CD8+ cells abolished the protective effect of dydrogesterone on the abortion rate in stressed mice by affecting the Th1/Th2 cytokine profile

To investigate whether the protective effect of progesterone substitution with dydrogesterone may be mediated by CD8+ cells, we specifically depleted this cell population using a mAb (33). As presented in Fig. 1B, we observed that the decrease of stress-triggered abortion in dydrogesterone-treated mice could be significantly abrogated if these mice have been CD8-depleted.

### Table II. The absolute numbers of cells isolated from the uterus and analyzed by flow cytometry

<table>
<thead>
<tr>
<th>Treatment of Mice</th>
<th>Daysa</th>
<th>Total Uterus Cell (×10⁶)bc</th>
<th>TNF-α⁺CD8+b</th>
<th>IFN-γ⁺CD8+b</th>
<th>IL-12⁺CD8+b</th>
<th>IL-4⁺CD8+b</th>
<th>Th1/Th2d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.5</td>
<td>6.2 ± 0.5</td>
<td>0.9 ± 0.1</td>
<td>1.3 ± 0.4</td>
<td>1.1 ± 0.2</td>
<td>0.9 ± 0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>Stress</td>
<td>7.5</td>
<td>6.7 ± 0.7</td>
<td>1.6 ± 0.3</td>
<td>1.6 ± 0.5</td>
<td>1.3 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td>2.3</td>
</tr>
<tr>
<td>Stress/dydrogesterone</td>
<td>9.5</td>
<td>6.3 ± 0.5</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>0.9 ± 0.2</td>
<td>1.7 ± 0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Control</td>
<td>9.5</td>
<td>6.3 ± 0.6</td>
<td>0.3 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.6 ± 0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Stress</td>
<td>9.5</td>
<td>6.4 ± 0.6</td>
<td>0.3 ± 0.2</td>
<td>1.1 ± 0.3</td>
<td>1.0 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>4.0</td>
</tr>
<tr>
<td>Stress/dydrogesterone</td>
<td>10.5</td>
<td>5.3 ± 0.7</td>
<td>1.3 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>3.0 ± 0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Stress/dydrogesterone</td>
<td>10.5</td>
<td>4.2 ± 0.7</td>
<td>0.3 ± 0.0</td>
<td>1.6 ± 0.5</td>
<td>0.5 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>10.5</td>
</tr>
</tbody>
</table>

a Days of gestation.
b Number of uterine cells have been calculated per mouse and are presented as the mean per group.
c The absolute numbers have been calculated per mouse and are presented as the mean per group.
d Th1/Th2 ratio has been calculated by adding the absolute numbers of CD8+/TNF-α+ cells to the number of CD8+/IFN-γ+ and CD8+/IL-12+ cells, the sum was then divided by 3 to obtain the mean Th1 value. This value has been divided by the absolute number of CD8+/IL-4+ cells as Th2 value. Bold numbers in the “Stress” groups depict a significant change (p ≤ 0.05) to the “Control” groups for the respective gestational day. Bold numbers in the “Stress/dydrogesterone” groups reflect significant changes when compared to the “Stress” groups. Significance of difference was analyzed by the Mann-Whitney U test.

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FIGURE 5. Flow cytometry to detect IFN-γ-positive decidual lymphocytes. Intracellular detection of IFN-γ in the uterine lymphocyte population on gestation days 7.5, 9.5, and 10.5 of the various experimental groups, again determined by flow cytometry. **, p < 0.01, as analyzed by the nonparametric Mann-Whitney U test.

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As mentioned earlier, Th2-like cytokines appear to contribute to the maintenance of pregnancy, whereas Th1 cytokines have been shown to be deleterious for pregnancy. In the present study, we observed that on gestation day 7.5 and 9.5, stressed mice showed a significantly increased percentage TNF-α⁺ uterine cells (Fig. 4). On gestation day 10.5, 5 days after stress exposure, levels of TNF-α were back to the percentage seen in control mice. Interestingly, injection of dydrogesterone significantly lowered the relative number of TNF-α⁺-producing uterine cells on days 7.5, 9.5, and even on 10.5. The absolute numbers of CD8⁺/TNF-α⁺ uterine cells are presented in Table II.

Further, we investigated the presence of the Th1 cytokines IFN-γ and IL-12 in uterus cells. As depicted in Fig. 5, nonstressed mice presented a significantly greater percentage of IFN-γ⁺ cells immediately after stress exposure, on gestation day 7.5, compared with stressed as well as stressed dydrogesterone-treated mice. However, on day 9.5, when the percentages in controls significantly decreased compared with day 7.5, this Th1 down-regulation
the dydrogesterone-induced down-regulation of the Th1 cytokines TNF-α, IFN-γ, and IL-12 present in uterus cells has been abolished in the CD8-depleted mice (Fig. 8A). Depletion of uterine CD8+ cells by injection of Lyt 2.1 has been confirmed by flow cytometry, as presented in Fig. 3B. Interestingly, the ratio between Th1/Th2 cytokines in stressed mice changed upon depletion of CD8 cells; Fig. 8B depicts the predominance of Th1 cytokine levels in stressed, dydrogesterone-treated and CD8-depleted mice with a ratio of 1, compared with a ratio of 0.4 in stressed, dydrogesterone-treated mice with no CD8 depletion.

Discussion
It is well known that progesterone mediates effects which are beneficial for the onset, development, and maintenance of pregnancy (10–14). In contrast, stress interferes with the course of pregnancy and may lead to abortions, putatively via decrease of progesterone (15, 16). Previous studies in our laboratory have demonstrated that stress-triggered abortion can be therapeutically approached by application of a progesterone derivative (31).

In this study, we now deliver evidence that stress exposure induces a decrease of uterine CD8+ cells within 24 h. This decrease can be restored by application of the progesterone derivative dydrogesterone, an observation which is clearly in agreement with the observations of a pregnancy protective effect of dydrogesterone treatment with respect to the abortion rate is almost abolished, thus, we conclude that the pregnancy protective effect of progesterone and dydrogesterone is predominantly mediated by uterine CD8+ cells (Fig. 9).

Interestingly, the population of uterine CD8+ cells changes subsequently (gestation days 9.5 and 10.5) from pregnancy protective to abortogenic, suggesting the presence of different functional subpopulations of uterine CD8+ cells throughout gestation. Functional CD8 is a dimeric protein made of either two α-chains (CD8αα) or an α-chain and a β-chain (CD8αβ) (35–37). Importantly, CD8αα and CD8αβ are differentially expressed on a variety of lymphocytes. CD8αβ is expressed predominantly on the surface of αβ T cells and thymocytes (38–41). Expression of CD8αα has been identified on a subset of γδ TCR+ intestinal intraepithelial lymphocytes, NK cells, dendritic cells, and a small fraction of CD4+ T cells, but αα-chains are essentially absent on cells of the circulation (41–44). The differential distributions of CD8αα and CD8αβ suggest that these two forms of CD8 are likely to mediate distinct functions. Based on the experimental set-up in our present study, we cannot differentiate whether the CD8 cells express an αα or αβ-chain. However, from preliminary results, we know that ~25% of uterine CD8 cells also express CD11c, thus belonging to the family of uterine dendritic cells. The percentage of CD8+/CD11c+ cells slightly diminished with stress and could be restored by dydrogesterone application (data not shown).

Gorzynski et al. (45) suggested that CD8+ γδ T suppressor cells and CD8αα indoleamine 2,3 dioxygenase-producing suppressor cells could be stimulated by CD200+CD8+ dendritic cells in stressed mice treated with dydrogesterone, compared with mice that were CD8-depleted in addition to stress and dydrogesterone treatment. Data are represented as the mean ± SEM. *, p < 0.05; **, p < 0.01, as evaluated by nonparametric Mann-Whitney U test.

FIGURE 6. Stress or dydrogesterone do not significantly alter levels of uterine IL-12. Percentage of IL-12-positive uterine lymphocyte populations from nontreated, stressed, and dydrogesterone-treated, stressed mice on gestation days 7.5, 9.5, and 10.5. Bars show means ± SEM. Data were analyzed by nonparametric Mann-Whitney U test.

FIGURE 7. Dydrogesterone treatment up-regulates IL-4 in uterus cells. Percentage of IL-4-positive uterine lymphocyte population of the various experimental groups on days 7.5, 9.5, and 10.5 of gestation. Data are represented as the mean ± SEM. *, p < 0.05; **, p < 0.01, as evaluated by nonparametric Mann-Whitney U test.

FIGURE 8. Depletion of CD8 abrogates the pregnancy protective effect of dydrogesterone treatment via an increase of proinflammatory cytokines and the Th1/Th2 ratio. A. Percentage of uterine lymphocyte populations positive for the cytokines TNF-α, IFN-γ, IL-12, IL-4, and IL-10 in stressed mice treated with dydrogesterone. Data are represented as the mean ± SEM. *, p < 0.05; **, p < 0.01, as evaluated by nonparametric Mann-Whitney U test. B. Th1/Th2 ratio has been calculated as follows: sum of percentages for TNF-α+, IFN-γ+, and IL-12+ cells, divided by 3 (to obtain the mean Th1 value). The sum of percentages for IL-4+ and IL-10+ uterine cells was calculated and divided by 2 (to obtain the mean Th2 value). The Th1/Th2 ratio has been obtained by dividing the mean Th1 value by the mean Th2 value.
cells. These CD8\(^+\) cells represent an important subset for the induction of cytokines such as IL-4, IL-10, and TGF-\(\beta\) that inhibit macrophages, TCR \(\alpha\beta\) cells and NK or NK \(\gamma\delta\) T cells, which produce IFN-\(\gamma\), TNF-\(\alpha\), and IL-12 and induce placental hemorrhage by up-regulation of \(\gamma\delta\) prothrombinase (25). However, CD8 depletion did not entirely abrogate the protective effect of progesterone and mimicked an abortion rate as seen in the stressed mice. This could be due to cellular infiltration of the fetomaternal interface by progesterone receptor-negative macrophages and NK cells, which are also capable of producing high levels of abortogenic cytokines. Another possible reason may be the existence of a T-reg cells, which are also capable of producing high levels of abortogenic cytokines. Another possible reason may be the existence of a T-reg cells, which are also capable of producing high levels of abortogenic cytokines.

In our present study using flow cytometry, we observed that the population of TNF-\(\alpha\) \(\pm\) uterus cells increases after stress exposure, compared with the control group. This stress effect can be counteracted by hydrogesterone treatment. This result confirms the notion of Th1 cytokines deleterious for pregnancy maintenance (21). Indeed, other Th1 cytokines, e.g., IFN-\(\gamma\), increased after stress exposure in murine matings (23, 50). Interestingly, Tangri and Raghuopathy (51) demonstrated that the placenta of an abortion-prone mating such as CBA/J \(\times\) DBA/2J produced high levels of TNF-\(\alpha\) and IFN-\(\gamma\), whereas in low abortion mating, e.g., CBA/J \(\times\) BALB/c, IL-10 is dominant. However, based on recent publications, the Th1/Th2 paradigm has been frequently challenged of late (52, 53), pointing toward the importance for future research in reproductive immunology to deliver additional experimental evidence.

In our present experiments, the percentage of IL-12-producing uterus cells did not change over the days investigated in the various groups, bringing into question the function of IL-12 as a Th1 inducer on the uterine level. We suggest that the physiologic function of IL-12 in pregnancy may rather be around the time of blastocyst adhesion, and additional studies are necessary to elucidate the role of this cytokine in the regulation of the immune system during pregnancy maintenance.

In conclusion, our data imply that stress leads to increased abortions by an alteration of the hormonal system with a consecutive dysregulation of the Th1/Th2 ratio via CD8\(^+\) cell-dependent pathways. Progesterone substitution by dydrogesterone can abrogate this effect by inducing a pregnancy protective Th2-biased immune response in the decidua.

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References
