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The Link between the PDL1 Costimulatory Pathway and Th17 in Fetomaternal Tolerance

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Fetomaternal tolerance has been shown to depend both on regulatory T cells (Tregs) and negative signals from the PD1–PDL1 costimulatory pathway. More recently, IL-17–producing T cells (Th17) have been recognized as a barrier in inducing tolerance in transplantation. In this study, we investigate the mechanisms of PDL1-mediated regulation of fetomaternal tolerance using an alloantigen-specific CD4+ TCR transgenic mouse model system (ABM-tg mouse). PDL1 blockade led to an increase in embryo resorption and a reduction in litter size. This was associated with a decrease in Tregs, leading to a lower Treg/effector T cell ratio. Moreover, PDL1 blockade inhibited Ag-specific alloreactive T cell apoptosis and induced apoptosis of Tregs and a shift toward higher frequency of Th17 cells, breaking fetomaternal tolerance. These Th17 cells arose predominantly from CD4+Foxp3− cells, rather than from conversion of Tregs. Locally in the placenta, similar decrease in regulatory and apoptotic markers was observed by real-time PCR. Neutralization of IL-17 abrogated the anti-PDL1 effect on fetal survival rate and restored Treg numbers. Finally, the adaptive transfer of Tregs was also able to improve fetal survival in the setting of PDL1 blockade. This is to our knowledge the first report using an alloantigen-specific model that establishes a link between PDL1, Th17 cells, and fetomaternal tolerance. The Journal of Immunology, 2011, 187: 000–000.

Accepted of the semiallogeneic fetus by the mother during pregnancy represents a physiologic model of in vivo immune tolerance (1, 2). Several mechanisms have been reported to take part in this complex interplay. More recently, the importance of regulation and trafficking of regulatory T cells at the fetomaternal interface has been investigated, even though its mechanisms remain a matter of debate (3). Because tolerance has been reconsidered as a balance rather than an all-or-none phenomenon (4), a number of studies both in transplantation and in fetomaternal mouse models are focusing on the role of regulatory T cells (Tregs), effector T cells (Teff), and costimulatory pathways and their reciprocal influence in balancing the immune response (5–7).

The PD1–PDL1 negative costimulatory pathway has been demonstrated to be critical for peripheral transplantation tolerance due to its ability to alter the balance between pathogenic T cells and Tregs (8, 9). Expression of PDL1 in donor tissue was crucial for the prevention of chronic rejection in a heart transplant model (10) and for the maintenance of tolerance at the uteroplacental interface (11). In a bone marrow transplantation model, both in vivo and in vitro, PD1–PDL1 costimulation blockade resulted in an increased lethality of graft-versus-host disease by increasing donor IFN-γ production and enhancing T cell alloimmune responses (12). In a diabetes model, PDL1 expression on the surface of islets provided protection against immunopathological injury after transplantation (13).

Our group has previously shown that the PD1–PDL1 negative costimulatory pathway plays a key role in inducing and maintaining fetomaternal tolerance in a mouse model. In particular, it was shown by Habicht et al. (14) that the expression of PDL1 on the surface of Tregs is essential to exert their suppressive effect and to control the maternal immune response. In fact, blocking PDL1 resulted in a loss of regulatory function and reduction in fetal survival rate. It has also been suggested that a deficiency of PDL1, in and of itself, may influence Teff and drive the balance toward fetal rejection by favoring Th1 and Th17 cell development and expansion (14). Consistent with that, several authors have reported the failure of the Th1/Th2 paradigm in fully explaining fetal rejection in different murine models (15–17) and allograft rejection in transplantation (18). It has also been recently observed that Th17 expansion is critical to initiate an alloimmune response, particularly in a Th1-deficient environment. Although no data are available in the allogeneic pregnancy model, Th17 expansion represents a barrier to establish tolerance (19, 20).

The aim of the current study was to investigate the mechanisms by which PDL1 mediates the interaction between Tregs and Teff and thereby directly affects the Th1/Th2/Th17 balance, leading to regulation at the fetomaternal interface. For this purpose, we took advantage of an alloantigen-specific CD4+ TCR transgenic mouse model system (ABM-tg mouse) to track alloantigen-specific cells generated during the immune response in a single class II mismatch murine model of fetomaternal tolerance.

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Abbreviations used in this article: dpc, days postconceptus; IP-10, IFN-γ–induced protein-10; RT-PCR, real-time polymerase chain reaction; Teff, effector T cell; tg, transgenic; Treg, regulatory T cell.

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Materials and Methods

Mice

B6.PL-Thy1.2/CyJ (Thy1.1 B6) and B6.C-H-2Bm12/KHeg (Bm12) mice were purchased from The Jackson Laboratory. ABM (anti-Bm12) mice were generated as previously reported (21) and maintained as a breeding colony in our animal facility. Foxp3 knock-in (Foxp3-GFP) mice were a kind gift of Dr. Rudensky (University of Washington). ABM Tg.Foxp3-GFP mice on B6 background were generated by crossing ABM mice with Foxp3 knock-in mice for eight generations in our own facility. All mice were housed in accordance with institutional and National Institutes of Health guidelines.

Treatment protocol

Pregnant female Thy1.1 B6 mice were injected i.p. with the blocking anti-mouse PDL1 mAb (M1H6) as previously reported (21). Neutralizing IL-17 mAb (MAB421; R&D Systems, Minneapolis, MN) has recently been described (20) and was administered i.p. at a dose of 84 μg on 4.5, 5.5, 6.5, 7.5, 8.5, and 11 days postcoitus (dpc).

Timed matings and resorption rates

Females were inspected daily for vaginal plugs, and sighting of a vaginal plug was designated as day 0.5 of pregnancy. Plugged females were either sacrificed between 11.5 and 13.5 dpc to examine the number of implantations and resorbing sites or were monitored until parturition and the plug was designated as day 0.5 of pregnancy. Plugged females were either sacrificed with 1 mg/ml collagenase at 37°C. Single-cell suspension was prepared by passing through the wire mesh. The cells were washed and resuspended in two Percoll fractions at densities of 1.095 and 1.030. The cells in the 1.030 fraction were underlayered with cells in the 1.095 density fraction. The cells were spun at 2500 rpm for 30 min. Cells were taken from the interface, washed, and counted for subsequent flow staining. Pan leukocytes (CD45) expressing Foxp3, IFN-γ, or IL-17 were stained on a FACSCalibur (Becton Dickinson, San Jose, CA), and cells were analyzed using FlowJo software.

ELISPOT assay

Splenocytes from pregnant mice (female Thy1.1 B6 mated with Bm12 male) were obtained as single-cell suspensions and used as responder cells (5 × 10^6). Splenocytes from male Bm12 mice were irradiated and used as stimulator cells. The ELISPOT assay was adapted to detect IFN-γ, IL-4, and IL-17-secreting cells as described previously (14).

Luminex cytokines assay

For cytokine analysis, splenocytes harvested at 11.5 and 13.5 dpc from pregnant Thy1.1 B6 mice were restimulated with irradiated Bm12 splenocytes. The cell-free supernatants of individual wells were removed after 48 h and analyzed by a multiplexed cytokine bead-based immunoassay (20).

Placenta intracellular cytokine staining

Placentae were collected at 13.5 dpc and were cut into small pieces and digested with 1 mg/ml collagenase at 37°C. Single-cell suspension was prepared by passing through the wire mesh. The cells were washed and resuspended in two Percoll fractions at densities of 1.095 and 1.030. The cells in the 1.030 fraction were underlayered with cells in the 1.095 density fraction. The cells were spun at 2500 rpm for 30 min. Cells were taken from the interface, washed, and counted for subsequent flow staining. Pan leukocytes (CD45) expressing Foxp3, IFN-γ, or IL-17 were stained on a FACSCalibur (Becton Dickinson, San Jose, CA), and cells were analyzed using FlowJo software.

Treg conversion in vitro

Thy1.2 Foxp3-GFP T cells from ABM Tg.Foxp3-GFP mice, generated in our laboratory by mating ABM tg mice with Rudensky reporter mice (B6 Foxp3-GFP knock-in), were used for in vitro studies. Isolated Foxp3-GFP knock-in CD4^+ T cells (1 × 10^6) were cocultured with syngeneic CD11c^+ cells (2 × 10^5), soluble anti-CD28 (1 μg/ml), soluble TGF-β (1 ng/ml), and plate-coated with anti-CD3 (1 μg/ml), in the presence or absence of anti-PDL1 mAb (1, 10, or 50 μg/ml). After 4 d in culture, the percentage of CD4^+Foxp3^+ cells was assessed by flow cytometry. We also collected the supernatant of these cultures at 48 h to evaluate the production of IL-17 by Luminex. Finally, CD4^+GFP^+ cells (0.2 × 10^6) from ABM Foxp3-GFP knock-in TCR-tg mice were cocultured with syngeneic CD11c^+ cells (0.05 × 10^6) and irradiated Bm12 cells (0.2 × 10^6) in the presence or absence of anti-PDL1. Production of IL-17 was evaluated after 48 h of culture. Cell isolation was performed by FACS-sorting CD4^+GFP^-, CD4^+GFP^+ cells, and CD11c^+ cells, resulting in greater than 99% purity.

RNA extraction and RT-PCR

RNA extraction was performed according to the manufacturer’s instructions (Invitrogen). Expression was measured as copies of any given gene divided by copies of the housekeeping gene GAPDH as previously described (14).

Statistics

Kaplan–Meier survival graphs were constructed, and a log rank comparison of the groups was used to calculate p values. Student t test was used for comparison of means between experimental groups examined by FACS analysis or ELISPOT assay. Differences were considered to be significant at p values <0.05.

Results

Reduction in fetal survival with PDL1 blockade correlates with a decrease in Tregs and Tregs/Teff ratio in the periphery and a lowering of expression of regulatory markers at the uteroplacental interface

To investigate the mechanisms by which the PD1–PDL1 costimulatory pathway affects fetomaternal tolerance, we took advantage of the ABM-tg mouse (21) to track alloantigen-specific CD4^+ cells
with either an effector or a regulatory phenotype development in a Thy1.2 B6 pregnant mouse previously mated with a Bm12 male (see Materials and Methods). We have previously shown that PDL1 is expressed at the fetomaternal interface and that blockade of this molecule resulted in significantly reduced fetal survival rate in a fully allogeneic (CBA × B6) mating setup (11). In this study, we report that PDL1 blockade remarkably increased fetal resorption (34 ± 10 versus 1.4 ± 10%, n = 7, p < 0.0001) and resulted in a reduced litter size compared with control IgG (5.8 ± 0.4 versus 8.5 ± 1, n = 7, p < 0.0001) in the Bm12 × B6 mating as well.

Subsequently, we adoptively transferred Thy1.2'ABM-tg cells at day 4 after detection of vaginal plug and treated the female mice with either anti-PDL1 mAb or control IgG. We analyzed samples at 11.5 and 13.5 dpc. Flow cytometry analysis demonstrated an increase in the percentage of Thy1.2'Teff (Thy1.2'CD44hiCD62Llow) in spleens of animals treated with anti-PDL1 compared with control IgG (72 ± 1.8 versus 65 ± 1.7%, p < 0.01), particularly at 11.5 dpc, whereas no differences were found at day 13.5 (56 ± 2.7 versus 52 ± 2.8%, p = 0.2). Absolute numbers did not show any difference either at day 11.5 (216,000 ± 25 versus 206,500 ± 13, respectively, p = 0.7) (Fig. 2A) or at day 13.5 (215,000 ± 55 versus 218,400 ± 33, p = 0.9). As shown in Fig. 2B, Thy1.2'Tregs (Thy1.2'CD25'Foxp3') were also significantly decreased after PDL1 blockade at the same time point in lymph nodes (data not shown) and spleen compartments in both percentage (13 ± 2.7 versus 4 ± 0.7%, p = 0.01) and absolute numbers of cells (382,200 ± 30 versus 217,500 ± 33, p = 0.01), whereas a less evident reduction was detectable at 13.5 dpc (5.8 ± 0.3 versus 3.9 ± 0.65%, p = 0.03, and 305,300 ± 28 versus 221,595 ± 22, p = 0.048), suggesting a role for this costimulatory pathway in actively regulating the balance between alloantigen-specific Tregs and Teff during the tolerance process in the periphery. In fact, analysis of Tregs/Teff ratio showed a reduction in percentage values at 11.5 dpc both in lymph nodes (0.5 ± 0.03 versus 0.2 ± 0.02%, p < 0.0002) and spleen (0.14 ± 0.03 versus 0.05 ± 0.008%, n = 11, p < 0.03) that was confirmed by calculating absolute numbers of cells in spleens (1.7 ± 0.2 versus 1 ± 0.1 on controls, respectively, p = 0.04) (Fig. 2C). These results suggest that a significant decrease in Thy1.2'Tregs rather than an increase in Thy1.2'Teff plays a major role in affecting the balance of the immune response.

Prior studies have also reported that PDL1 blockade can affect regulatory markers locally in the placenta (11, 22). In line with this, we observed the expression of some of the regulatory genes detected by RT-PCR at the fetomaternal interface is diminished in animals treated with anti-PDL1 compared with controls. In particular, Foxp3 (0.0002 ± 0.0002 versus 0.5310 ± 0.2042 copies/copies GAPDH, p < 0.05), IDO (0.31 ± 0.13 versus 1.08 ± 0.24 copies/copies GAPDH, p < 0.04), and ICOS (0.0001 ± 0.00000000004 versus 0.44 ± 0.15 copies/copies GAPDH, p < 0.03) were downregulated in anti-PDL1–treated groups (Supplemental Fig. 1).

These data confirm that blocking the PD1–PDL1 negative costimulatory pathway interferes with the fine balance between Teff, Tregs, and regulatory molecules in the periphery as well as at the uteroplacental interface.

PDL1 blockade is associated with a switch in Th balance toward Th17

To investigate whether PD1–PDL1 costimulation is able directly to affect Teff generation and/or tip the Th1/Th2/Th17 balance to promote tolerance during pregnancy, we first analyzed the cytokine profile both locally in the placenta with intracellular cytokine flow staining/RT-PCR and in the periphery with ELISPOT/Luminex-based assays. The most interesting finding was that blocking PDL1 resulted in a major inhibition of Th1 cytokine production both in the periphery (ELISPOT: IFN-γ 24.33 ± 4.717 for anti-PDL1–treated mice versus 141.8 ± 29.56 spots per 0.5 × 106 splenocytes of control IgG-treated mice, p < 0.0002; Luminex: IFN-γ–induced protein-10 (IP-10) 20.38 ± 4.8 versus 42.5 ± 7.0 pg/ml, respectively, p < 0.02; TNF-α 51.45 ± 25.67 versus 190.3 ± 12.98 pg/ml, respectively, p < 0.007) and at the uteroplacental interface (TNF-α 0.000002 ± 0.000002 versus 1.17 ± 0.41 copies/copies GAPDH, respectively, p < 0.05) with an increase in the production of Th2 cytokines (IL-4 and IL-5) as reported in Fig. 3. Similar results were obtained at the second time point 13.5 dpc for Th1 cytokines (ELISPOT: IFN-γ 91.2 ± 25 for control IgG-treated mice versus 39 ± 6.7 spots per 0.5 × 106 splenocytes of anti-PDL1–treated mice, p = 0.012; Luminex: IP-10 42.5 ± 7 versus 20.38 ± 4.8 pg/ml, respectively, p = 0.019; TNF-α 29.9 ± 6.4 versus 9.53 ± 1.9 pg/ml, respectively, p < 0.001) as well as for Th2 cytokines (ELISPOT: IL-4 88.8 ± 8.6 for control IgG-treated mice versus 114 ± 3.6 spots per 0.5 × 106 splenocytes of anti-PDL1–treated mice, p = 0.018; Luminex: IL-4 21.67 ± 3.4 versus 40.67 ± 6 pg/ml, respectively, p = 0.02; IL-5 51.1 ± 25 versus 153.4 ± 73 pg/ml, respectively, p = 0.010). Notably, we detected a significant increase in IL-6 production at 11.5 dpc and later at 13.5 dpc in anti-PDL1–treated animals compared with controls (11.5 dpc: 285.4 ± 51.7 versus 27.13 ±

**FIGURE 1.** PDL1 blockade significantly increases fetal resorption and results in reduced litter size. Thy 1.1 B6 females were mated with Bm12 males, and at days 6.5, 8.5, 10.5, and 12.5, anti-PDL1 mAb or control IgG was injected. Some mice were observed for fetal resorption at 13.5 dpc and others were allowed to go to term and litter sizes counted. A, Scattered dot plots depicting percentages of embryos resorbing in mice treated with anti-PDL1 (n = 7) or control IgG (n = 7). B, Scattered dot plots depicting litter size in mice treated with anti-PDL1 or control IgG are shown.
3.3 pg/ml, respectively, \( p < 0.0002 \); 13.5 dpc: 1579.6 versus 741.8 pg/ml, respectively, \( p < 0.03 \). As it has been reported that IL-6 represents a differentiation factor for Th17 and a target for IL-17–producing cells (23), we examined IL-17 production both locally and in the periphery. As anticipated, ELISPOT and Luminex assays showed a significant increase in the level of IL-17 in the periphery in anti-PDL1–treated mice compared with controls (Fig. 4A). This increase in IL-17 was also demonstrable locally at the uteroplacental interface as evidenced by intracellular flow cytometry staining (Fig. 4B) and by RT-PCR of the placentae of anti-PDL1–treated mice versus control IgG-treated mice (1.1 ± 0.1 versus 0.3 ± 0.05%, \( p < 0.005 \), and 10.36 ± 3.3 versus 1.87 ± 1.2 copies/copies GADPH, \( p < 0.03 \), as shown in Fig. 4B).

Neutralization of IL-17 abrogates the anti-PDL1 effect on fetal survival rate

Having found differences in Tregs as well as Th17 in our ABM-tg pregnancy model, we focused on delineating the interplay, if any, between Th17 and downmodulation of Tregs in this model. To determine this, we first set up matings between Bm12 males and B6 Thy1.1 females and then treated them with anti-PDL1 alone or in...
combination with IL-17 neutralizing Ab. Neutralization of IL-17 in anti-PDL1-treated animals showed a significant increase in litter size compared with anti-PDL1 alone (8.6 ± 0.74 versus 4.7 ± 0.14, n = 5 and n = 7, respectively, p < 0.0001) and a significant decrease in fetal resorption (16.2 ± 3.4 versus 37.4 ± 1.7%, n = 5 and n = 7, respectively, p < 0.0002), as represented in Fig. 5A and 5B. We then tested the role of blocking PDL1 in IL-17−/− mice mated with Bm12 males and observed no difference in litter size (8 ± 0.4 versus 8.5 ± 0.8, n = 5, respectively, p = ns) as well as in embryo resorption rate (14.8 ± 4 versus 15.7 ± 2%, n = 5, respectively, p = ns) when animals were treated with anti-PDL1 compared with control IgG (Fig. 5C, 5D). This provided evidence for an important role for IL-17–producing cells in interfering with tolerance induction in this experimental model.

Neutralization of IL-17 significantly reduces anti-PDL1–mediated expansion of Teff and abrogates the anti-PDL1–mediated effect in reducing Treg numbers

Our next step was to determine the effect of neutralization of IL-17 in anti-PDL1–treated animals on adoptively transferred Thy1.2+ ABM-tg cells. While the expansion of Thy1.2+Teff is slightly increased in anti-PDL1–treated animals upon IL-17 neutralization, there is a more marked effect in maintaining Thy1.2+Tregs compared with administration of anti-PDL1 alone (Fig. 6A, 6B). This results in a relatively increased Treg/Teff ratio compared with that of the group treated with anti-PDL1 alone (Fig. 6C).

PDL1 blockade inhibits Ag-specific alloreactive T cell apoptosis and induces apoptosis of Tregs

Because one of the most important mechanisms shown to induce and/or maintain fetomaternal tolerance is the apoptosis of activated maternal cells (2, 24), we investigated its possible link with the PDL1–PDL1 costimulatory pathway. We first focused our attention on the apoptosis of alloantigen-specific ABM-tg cells. Flow cytometry analysis at 11.5 dpc showed a significant difference in anti-PDL1 compared with control IgG (Fig. 7A, 7B). These data were corroborated by expression of apoptotic markers locally in the placenta by RT-PCR. RT-PCR analysis showed a significant reduction in DR5, a receptor involved in the TRAIL pathway for apoptosis, in the placentae of anti-PDL1–treated mice versus control IgG (Fig. 7C, 7D, 7E, 7F).
Abs and detected by flow cytometry.

Methods

Placentae were collected at 13.5 dpc and were digested with collagenase at 37˚C and processed to obtain infiltrating cells as described in

expression was determined in anti-PDL1 mAb or control IgG Ab group by RT-PCR or by flow cytometry. For placenta intracellular cytokine staining,

mice were irradiated and used as stimulator cells. The ELISPOT assay was performed to detect IL-17–secreting cells. Splenocytes from pregnant Thy1.1 B6 mice were also restimulated with irradiated Bm12 splenocytes, and the cell-free supernatants of individual wells were removed after 48 h and analyzed by a multiplexed cytokine bead-based immunoassay for IL-17 production. Data are representative of three independent experiments using at least 3 mice per group.

A

B

IL-17 ELISPOT (Spleen)

IL-17 Luminex (Spleen)

IL-17 RT-PCR (Placenta)

IL-17 Flow (Placenta)

FIGURE 4. Anti-PDL1 treatment results in higher expression of IL-17 both in the periphery and locally in the placenta. Splenocytes from pregnant mice (female Thy1.1 B6 mated with Bm12 male) were obtained as single-cell suspensions and used as responder cells (5 x 10⁶). Splenocytes from male Bm12 mice were irradiated and used as stimulator cells. The ELISPOT assay was performed to detect IL-17–secreting cells. Splenocytes from pregnant Thy1.1 B6 mice were also restimulated with irradiated Bm12 splenocytes, and the cell-free supernatants of individual wells were removed after 48 h and analyzed by a multiplexed cytokine bead-based immunoassay for IL-17 production. Data are representative of three independent experiments using at least 3 mice per group. In parallel, placenta were removed, and IL-17 expression was determined in anti-PDL1 mAb or control IgG Ab group by RT-PCR or by flow cytometry. For placenta intracellular cytokine staining, placenta were collected at 13.5 dpc and were digested with collagenase at 37˚C and processed to obtain infiltrating cells as described in Materials and Methods. Pan leukocytes (CD45) expressing IL-17 were stained on the surface with anti-CD45 FITC and intracellularly with anti–IL-17 allophycocyanin Abs and detected by flow cytometry. B, Left panel, Scatter plots depicting expression of IL-17 cytokine by RT-PCR at the fetomaternal interface of pregnant mice treated as above. Middle panel, Bar graphs depicting detection of IL-17 cytokine by intracellular cytokine staining at the fetomaternal interface of pregnant mice treated as above, with representative flow plots in each treatment group shown on the right. The data represent the mean ± SD of the results obtained from three separate experiments.

0.16 versus 1.58 ± 0.14 copies/copy GADPH, p < 0.001). These data indicate a role for PDL1 blockade in inhibiting the apoptosis of activated alloreactive T cells at the periphery and limiting the availability of molecules involved in the apoptotic process.

We next explored if PDL1 blockade is responsible for inducing apoptosis of Tregs and if anti–IL-17 coadministration affects Treg apoptosis. We observed that apoptosis of Thy1.2+Tregs (Thy1.2+ CD4+CD25+Foxp3+) was increased in anti-PDL1-treated animals compared with controls (489.084 ± 51 vs. 204,971 ± 37, p < 0.001) and compared with groups treated with either a combination of anti-PDL1 and anti–IL-17 or anti–IL-17 alone (227,399 ± 43 and 126,638 ± 10, respectively, p < 0.02 and p < 0.002, respectively) as shown in Fig. 7C.

PDL1 blockade reduces Tregs and increases the conversion of CD4+Foxp3+ cells into IL-17–producing T cells

To determine if PDL1 blockade prevents induction of Tregs, shifts Tregs to Th17 producers, or both, we performed in vitro cultures of CD4+Foxp3+ or CD4+Foxp3+ cells from Foxp3 knock-in mice in the presence or absence of anti-PDL1 mAb. Our in vitro data show that anti-PDL1 decreases conversion of CD4+Foxp3+ cells into CD4+Foxp3+ Tregs. This decrease in conversion is small but significant (p < 0.01) (Fig. 8A). Moreover, PDL1 blockade increases the frequency of IL-17–producing cells from CD4+Foxp3+ cells in a dose-dependent manner (Fig. 8B). However, when CD4+Foxp3+ Tregs were cultured in the presence of anti-PDL1 mAb, they failed to convert to Th17-producing cells (Fig. 8C).

To strengthen our in vitro findings, we used ABM Tg.Foxp3-GFP mice in which Ag-specific Tregs (CD4+Thy1.2+Foxp3-GFP+) can be detected, isolated, and adoptively transferred. Flow-sorted Thy1.2+CD4+Foxp3+Foxp3-GFP+ (Tregs) and GFP- cells (0.3 x 10⁶) were injected i.p. into Thy 1.1 B6 female mice mated with Bm12 males and treated with either anti-PDL1 or control IgG. We then harvested these cells 72 h after injection to evaluate their fate (Supplemental Fig. 2). About 15–20% of injected cells were present on secondary lymphoid organs 3 d after injection (Fig. 9A). Whereas both control and anti-PDL1–treated groups lost Foxp3 expression after transfer of Thy1.2+CD4+Foxp3-GFP+ cells, the group treated with anti-PDL1 showed a greater conversion to Foxp3-GFP− cells (Fig. 9B). Moreover, IL-17 production was very low on both groups, refuting the hypothesis of increased conversion of Foxp3-GFP+ cells into Th17 cells in the setting of PDL1 blockade (Fig. 9E). After transfer of Foxp3-GFP− cells, we observed about a 5% conversion to Foxp3-GFP− cells in
both groups (Fig. 9D). Notably, we noticed an increased conversion of Foxp3-GFP<sup>+</sup> cells into IL-17-producing T cells (Fig. 9F), in agreement with our in vitro data above. Overall, these data show that PDL1 blockade increases the conversion of Foxp3-GFP<sup>+</sup> cells into IL-17-producing cells and decreases the number of Tregs but does not seem to convert Tregs into IL-17-producing T cells.

To confirm the role of Tregs in PDL1-induced fetomaternal tolerance, we performed similar adoptive transfer of Thy1.2<sup>+</sup>Foxp3<sup>-</sup>GFP<sup>+</sup> (Tregs) and GFP<sup>+</sup> cells from ABM Tg.Foxp3-GFP mice into Thy1.1 B6 female mice mated with Bm12 males and treated with either anti-PDL1 or control IgG. However, this time we evaluated the pregnant B6 mice for litter size. A group of control mice without any adoptive transferred cells was also set up, and litter size of all groups was assessed. The litter size in Thy1.1 B6 females treated with anti-PDL1 mAb was close to six pups per litter. After transfer of Tregs, there was a significant increase in the litter size in this group to eight pups per litter (p = 0.0033) (Fig. 9G). There was no increase in the control IgG-treated group receiving Tregs versus not receiving any Tregs (Fig. 9G). This could presumably be because of no defect in the litter size in the control IgG group, and hence an additional dose of Tregs did not change the litter size outcome. This beneficial effect was specific to GFP<sup>+</sup> cells as transfer of GFP<sup>-</sup> cells into anti-PDL1 or control IgG-treated Thy1.1 B6 (mated with Bm12) mice did not result in rescue of phenotype in the anti-PDL1 group (litter size, 5.0 ± 0.71 in GFP<sup>-</sup> plus anti-PDL1 versus 5.0 ± 0.91 in no cells plus anti-PDL1 group; n = 4, p = ns). Litter size in GFP<sup>-</sup> cells plus control IgG and no cells plus control IgG was normal and close to eight pups per litter.

**Discussion**

In this report, we demonstrate that PDL1 blockade reduces fetal survival rate by affecting various immune cells and regulatory molecules both in the periphery and at the uteroplacental interface. For this purpose, we used an alloantigen-specific CD4<sup>+</sup> transgenic model with a Bm12 × B6 mating. First, PDL1 blockade inhibits the negative regulation of the immune response against alloantigens and contributes to fetomaternal tolerance by controlling alloantigen-specific Tregs in vivo in the periphery. The immunoregulatory role of the PD1–PDL1 costimulatory pathway in peripheral tolerance has previously been described in a fully allogeneic heart transplantation model (25) and in a class II mismatch model of skin transplantation (26). In both cases, PDL1 blockade resulted in an acceleration of allograft rejection. This was attributed to either a failure in immune regulation as a result of a decrease in Treg numbers and/or ineffective suppressive effect. Wang et al. (27) demonstrated that the deficiency of PDL1 molecules alters the balance of peripheral lymphocytes by decreasing the number of Tregs. In addition, there is an increase in the number of APCs that express a higher level of costimulatory molecules, thereby enhancing allogeneic immune responses in PDL1<sup>−/−</sup> recipients and accelerating heart allograft rejection (27). In this study, we did not look specifically at the APCs. Kitazawa

**FIGURE 5.** Neutralization of IL-17 abrogates anti-PDL1 effect in reducing fetal survival rate and increasing embryo resorption. Pregnant female Thy1.1 B6 mice were injected with the blocking anti-mouse PDL1 mAb. Neutralizing IL-17 mAb was administered to some of the mice treated with anti-PDL1 Ab. The degree of fetal resorption was determined at 13.5 dpc, and litter size was determined in a set of mice at term. Pregnant IL-17<sup>−/−</sup> mice were used for fetal resorption and litter size studies for some experiments. **A**, Scattered dot plots depicting percentages of embryos resorbing in mice treated with control IgG (n = 10), anti-PDL1 (n = 7), anti-PDL1 plus anti–IL-17 (n = 10), and anti–IL-17 alone (n = 9). **B**, Scattered dot plots depicting litter size in mice treated with control IgG (n = 7), anti-PDL1 (n = 7), anti-PDL1 plus anti–IL-17 (n = 5), and anti–IL-17 alone (n = 5). **C**, Scattered dot plots depicting percentages of embryos resorbing in IL-17<sup>−/−</sup> mice treated with control IgG (n = 4) and anti-PDL1 (n = 4). **D**, Scattered dot plots depicting litter size in IL-17<sup>−/−</sup> mice treated with control IgG (n = 4) and anti-PDL1 (n = 4).
et al. (28) have recently shown that the PD1–PDL1 pathway is required for Tregs effectively to suppress the alloreactive responses of Teff. In addition, PDL1 expression by dendritic cells is necessary for the induction of Foxp3+ adaptive Tregs both in vitro and in vivo (29). Finally, the PD1–PDL1 costimulatory pathway and Tregs have been demonstrated to participate strictly in the development of fetomaternal tolerance (14) and in the prevention of fetal rejection in an abortion-prone experimental model of murine pregnancy (30).

In contrast, our results document that PDL1 blockade also modulates the balance of alloimmune response locally at the fetomaternal interface with the involvement of different molecules and pathways. In line with this, Yang et al. (31) demonstrated that PDL1 blockade in Bm12 into B6 heart transplant significantly accelerates chronic rejection depending on the expression of PDL1 on donor tissue that regulates recipient alloimmune responses. Similarly, Tanaka et al. (10) described a shift in the balance between Tregs and Teff during PDL1 blockade in a fully allogeneic heart transplant due to an inhibition of the delivery of the negative costimulatory signal and also showed that PDL1 expression in donor tissue was essential to prevent graft infiltration and rejection.
Our results in the alloantigen-specific model also support the role for PD1–PDL1 costimulation in promoting Ag-specific Tregs and in limiting Ag-specific Teff expansion in the context of fetomaternal tolerance.

Furthermore, our data suggest that PD1–PDL1 costimulation also contributes to the maintenance of fetomaternal tolerance by promoting apoptosis of maternal Ag-specific activated cells. It has been reported earlier that the PD1–PDL1 costimulatory pathway plays an important role in fetomaternal tolerance by inducing apoptosis of paternal Ag-specific T cells and that PD1 action is the main mechanism that prevents T cell accumulation in lymph nodes (32). Even though the authors did not confirm directly a role for PDL1, they hypothesized that within the network of immunomodulatory pathways present at the fetomaternal interface, PD1–PDL1 is able to tip the maternal immune response toward a favorable milieu by limiting the accumulation of alloantigen-specific cells through apoptosis and modulation of cytokine secretion (32). In addition to that, a study from Bai et al. (33) on the TRAIL apoptotic pathway in humans showed that DR5 expression on cytotrophoblast cell surface promotes susceptibility to apoptosis and that lack of DR5 cell surface expression may direct TRAIL-induced cytotoxicity away from the placenta and toward the maternal system. This is in accordance with our observation that DR5 expression at the fetomaternal interface is reduced in anti-PDL1–treated animals and accounts for a diminished responsiveness to TRAIL-mediated apoptosis.

As part of PD1–PDL1 immunomodulatory function, our study also shows that PDL1 blockade is associated with a switch in Th
balance toward Th17, which leads to a reduction in fetal survival and increase in the fetal resorption rate in our single class II mismatch B6 × Bm12 mating model. Habicht et al. (14) described a similar increase in IL-17 production in the placenta of PDL1−/− females (on B6 background) mated with CBA males compared with B6 (wild-type) control female mated with CBA male. In the current study, we show in an Ag-specific model that the Th1 response is abolished for the most part in anti-PDL1–treated animals, and despite detecting a Th2 cytokine production, the development of a Th17 population overcomes it and creates a break in tolerance. Despite the recently discovered plasticity between Th17 and Tregs (34), the Th17 population in anti-PDL1–treated animals seems to arise from CD4+Foxp3− cells and not Tregs, as evident in our in vitro and adoptive transfer models (Figs. 8B, 9F).

It has previously been reported that successful pregnancy is a phenomenon mediated by Th2 cells and that Th1 cytokines such as IFN-γ and TNF-α may favor fetal loss (35). Chaouat et al. (36), on the contrary, shed light on the fact that some Th1 cytokines play a beneficial role in promoting implantation (IFN-γ) and that other new cytokines have been found to contribute to tipping the balance of immune response during pregnancy, such as IL-15, IL-18, IL-12, IL-16, and IL-27. However, whether these cytokines promote fetomaternal tolerance or not is not entirely clear.

Therefore, we focused mainly on confirming the role of IL-17 in contributing to fetal rejection and its complex interplay with both PD1–PDL1 costimulation and Tregs modulation in interrupting the state of tolerance. Our study demonstrates that neutralization of IL-17 abrogates the anti-PDL1 effect on fetal survival rate. It also confirms a detrimental role for IL-17–producing cells in promoting and sustaining fetal rejection as evidenced by the IL-17 knockout data. Our data with these mice indicate that IL-17–producing cells play a major role in disrupting the state of tolerance early on, but further studies are necessary to investigate the exact mechanism in detail.

Moreover, it has been shown that PD1 blockade in transplantation is correlated with a net increase in alloreactive T cells due to a reduction in apoptosis mechanisms. It has also been correlated with a reduction in Tregs and a decreased Treg suppressive effect (9, 26). In this study, we show that PD1 blockade is associated with a reduction in the absolute number of Tregs. This results in a failing suppressive effect on alloreactive T cells and in particular in those producing IL-17. Different mechanisms have been reported in an attempt to explain the link between PDL1 and Tregs. Francisco et al. (8) described a direct role for PDL1 in regulating induced Treg development and function. The authors showed that in the absence of PDL1, naive CD4+ T cells minimally convert to Tregs, and PDL1 itself enhances and sustains Foxp3 expression and the suppressive function of induced Tregs (8). Despite our initial in vitro findings suggesting similar effect, our adoptive transfer experiments in vivo did not show a significant decrease in conversion in the setting of PD1 blockade. Possible explanations for the divergent results might be related to the number of Tregs transferred, timing of analysis, or model used. Nonetheless, other authors have suggested that the expression of PDL1 on tumor cells exerts an antiapoptotic effect and renders them resistant to apoptosis (37, 38), which findings are in accordance with the observed increased apoptosis of Tregs in our results.

In summary, it has been shown that Tregs are able to prolong graft survival not only because of their suppression of Teff but also because of their anti-inflammatory properties (39). It has also been reported that Tregs represent a key mechanism in inducing fetal acceptance during pregnancy both in mice and humans (6, 40). PD1–PDL1 negative costimulation mediates the induction of peripheral tolerance both in a transplantation model as well as in a fetomaternal experimental model by favoring the regulatory function exerted by Tregs (9, 10, 13, 14, 30). In this study, we show that PD1–PDL1 negative costimulation is essential to maintain fetomaternal tolerance because of its role in limiting Teff expansion and promoting Tregs in an Ag-specific manner. We have demonstrated that tolerance at the fetomaternal interface results from the balance of different factors that facilitate the regulation of the immune response. In this context, we propose...
that skewing the response toward Th17 away from Tregs by inhibiting PDL1 costimulation can be crucial in breaking fetomaternal tolerance, and this effect seems to be mediated by a conversion of CD4+ Foxp3− cells to Th17 cells and increased apoptosis of Tregs. Furthermore, the neutralization of IL-17 or the adoptive transfer of Ag-specific Tregs is able to abrogate the deleterious effect of PDL1 blockade. Therefore, PDL1 expression may represent the key factor in balancing Tregs and Th17 cells and maintaining tolerance at the fetomaternal interface. Further studies are urged to assess a possible role for PD1–PDL1 co-stimulation in affecting the interplay between Tregs and Th17 in allograft rejection during transplantation.

**Disclosures**

The authors have no financial conflicts of interest.

**FIGURE 9.** Fate of Thy1.2+Foxp3-GFP cells after adoptive transfer and their effect on litter size. Flow-sorted ABM Thy1.2+Foxp3-GFP+ (Tregs) and Thy1.2+Foxp3-GFP− cells (0.3 × 10⁶) were injected i.p. into Thy 1.1 B6 female mice mated with Bm12 males and treated with either anti-PDL1 or control IgG. We then harvested these cells 72 h after injection to evaluate their fate. Some pregnant females transferred with Tregs were also followed long-term to evaluate effect on litter size. A, Representative flow cytometry plots of Thy1.2+ cells recovered from secondary lymphoid organs after transfer of Thy1.2+Foxp3-GFP+ cells (Ag-specific Tregs), with mean total cells obtained by multiplying total number of cells by the percentage of Thy 1.2+ cells (n = 3 to 5/group). B, Representative histograms of Foxp3 expression on Thy 1.2+ cells with mean percentage between groups after transfer of Tregs. C, Representative flow cytometry plots of Thy 1.2+ cells recovered from secondary lymphoid organs after transfer of Thy1.2+Foxp3-GFP− cells, with mean total cells of three animals per group. D, Representative histograms of Foxp3 expression on Thy 1.2+ cells with mean percentage between groups after transfer of Thy1.2+Foxp3-GFP− cells. E and F, IL-17 production by Luminex after restimulation with irradiated Bm12 cells 3 d after cell transfer of Thy1.2+Foxp3-GFP+ or GFP+, respectively. G, Average litter size in Thy1.1 B6 females mated with Bm12 males and adoptively transferred with Thy1.2+Foxp3-GFP+ cells or Thy1.2+Foxp3-GFP− or no cells after anti-PDL1 Ab or control IgG treatment (n = 4 mice per group).
References


Figure Legends:

Supplemental Figure 1: PDL1 blockade leads to reduction in various regulatory markers at the fetomaternal interface

Placentae were collected at day 13.5 dpc from anti PDL1 or control IgG treated Thy1.1 B6 females (mated with Bm12). Expression of various markers was determined by real time PCR. A. Scattered dot plots depicting expression of Foxp3 and IDO by RT-PCR at the fetomaternal interface of pregnant mice at 11.5 dpc treated with anti PDL1 or Control IgG. Data are representative of three independent experiments (n = 5 and n =7 respectively). B. Scattered dot plots depicting expression of PDL1 and ICOS by RT-PCR at the fetomaternal interface of pregnant mice at 11.5 dpc treated with anti PDL1 or Control IgG. Data are representative of three independent experiments (n = 5 and n =7 respectively).

Supplemental Figure 2: Adoptive transfer of Thy1.2\(^{+}\)Foxp3-GFP ABM TCR-tg T cells.

A, Representative flow cytometry plots of live gate and SSC doublets of ABM Tg CD4\(^{+}\)T cells (after positive magnetic sorting for CD4\(^{+}\) cells). B, Flow cytometry sorting of CD4\(^{+}\) Foxp3GFP\(^{+}\) (P1) and Foxp3GFP\(^{-}\) cells (P2). C, Allocation of sorted cells into 4 groups of pregnant Thy 1.1 B6 mice in combination of antibody treatment. Cells were then harvested 3 days after adoptive transfer as depicted on horizontal arrow.
Control Ig  anti PDL1

Copies Foxp3/copies GADPH

Copies IDO/copies GADPH

Copies PDL1/copies GADPH

Copies ICOS/copies GADPH

Supplemental Figure 1

A

B

control Ig anti PDL1

p<0.05

p<0.04

p<0.04

p<0.03
ABM Tg.Foxp3-GFP cells (Thy 1.2+)

Cell Sorting

CD4+Foxp3GFP+

CD4+Foxp3GFP−

Adoptive Transfer (300,000 cells)

Pregnant Thy1.1 B6 mice

IgG (n=3)

Anti-PDL1 (n=5)

IgG (n=3)

Anti-PDL1 (n=3)

Day 0 2 3

Harvesting

Ab Dose 0.5 mg