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A Critical Role for the Programmed Death Ligand 1 in Fetomaternal Tolerance

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Fetal survival during gestation implies that tolerance mechanisms suppress the maternal immune response to paternally inherited alloantigens. Here we show that the inhibitory T cell costimulatory molecule, programmed death ligand 1 (PDL1), has an important role in conferring fetomaternal tolerance in an allogeneic pregnancy model. Blockade of PDL1 signaling during murine pregnancy resulted in increased rejection rates of allogeneic concepti but not syngeneic concepti. Fetal rejection was T cell– but not B cell–dependent because PDL1-specific antibody treatment caused fetal rejection in B cell–deficient but not in *RAG-1*–deficient females. Blockade of PDL1 also resulted in a significant increase in the frequency of IFN-γ–producing lymphocytes in response to alloantigen in an ELISPOT assay and higher IFN-γ levels in placental homogenates by ELISA. Finally, PDL1-deficient females exhibited decreased allogeneic fetal survival rates as compared with littermate and heterozygote controls and showed evidence of expansion of T helper type 1 immune responses in vivo. These results provide the first evidence that PDL1 is involved in fetomaternal tolerance.

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The exact mechanisms of fetomaternal tolerance remain unknown. A role for HLA-G, Fas–FasL, or TRAIL-TRAIL-R in the apoptosis of maternal leukocytes during pregnancy has been documented in studies in mice and in humans (1–3). Expression of complement regulatory protein, crry, in the placenta also promotes fetomaternal tolerance (4). Indoleamine 2,3 dioxygenase (IDO) also has been shown to protect allogeneic concepti from maternal T cell–mediated immunity (5, 6). A recent report by Aluvihare et al. suggests a role for regulatory T cells in suppression of maternal allogeneic responses against the fetus (7).

Activation of T lymphocytes requires two signals, one delivered by the TCR complex after antigen recognition and one provided on engagement of costimulatory receptors. The costimulatory signal can be either posi-

tive or negative; the interplay between these signals may determine the fate of immune responses in vivo (8, 9). The inhibitory costimulatory molecule programmed death 1 (PD1) and its ligands, PDL1 and PDL2, play a role in regulating immune responses in vivo (8, 9), including acquired transplantation tolerance (10–14).

One of the interesting observations related to the PD1:PDL1/PDL2 pathway is that expression of the ligands is not restricted to BM-derived cells but has been reported in parenchymal cells in several tissues (15–18). In human placenta, PDL1 is expressed by villous syncytiotrophoblasts and cytotrophoblasts, the fetal cells that lie in close contact with maternal blood and tissue (17). Tissue expression of these ligands may play a critical role in regulating local immune responses in vivo (8, 9). We now provide the first evidence that PDL1 plays a critical role in fetomaternal tolerance.

The online version of this article contains supplemental material.

RESULTS AND DISCUSSION

Kinetics of expression of PD1 and PDL1 and PDL2 at the fetomaternal interface

In the first set of experiments, expression of PD1 and its ligands, PDL1 and PDL2, and CD28/CTLA-4 and their ligands, B7.1 and B7.2, were assessed in placentas of CBA females mated with C57BL/6 (B6) males. PDL1 and PDL2 expression was detected as early as 10.5 days post–conception (dpc) in all concepti, with a maximal expression for both ligands at d 13.5 dpc (Fig. 1, A and D), reduced expression at d 16.5 for PDL1 (Fig. 1 C), and lack of expression of PDL2 at this later time point (Fig. 1 F). PDL2 was expressed throughout the decidua (Fig. 1 B), whereas PDL1 expression was restricted to the decidua basalis, which is the maternal component of the placenta and is next to the trophoblastic giant cells (Fig. 1 A). This restricted expression suggests a role for PDL1 in regulating the maternal alloimmune response. Expression of PDL1 was negligible in the placental sections from syngeneic matings (CBA x CBA) at all the time points studied (unpublished data). B7.2 expression was maximal at 13.5 dpc (Fig. 1 C) and was negligible at 10.5 and 16.5 dpc. PD1, CTLA-4, CD-28, and B7.1 were not detected at all the time points studied.

Effect of blockade of costimulatory pathways on rate of spontaneous resorption in CBA x B6 matings

To study the role of the PD1 pathway in fetomaternal tolerance, we used an established model of allogeneic pregnancy; CBA x B6 matings (5, 6). The rate of spontaneous resorption in this model was 18% ($n = 30$), confirming published reports (5, 6). We then used blocking monoclonal antibodies to PDL1, PDL2, and B7.2 (ligands shown to be expressed in the placenta, see above) in this allogeneic pregnancy model. Control IgG–treated mice had a rate of spontaneous resorption similar to that in the unmanipulated control mice (Fig. 2). In vivo blockade with anti-PDL1 mAb resulted in significant increase in the rate of spontaneous fetal resorption (86%, $P < 0.0001$ by unpaired t test) (Fig. 2 A). However, blockade with anti-PDL2 or anti–B7.2 antibodies had no effect on the spontaneous resorption rate (Fig. 2 A). None of the antibodies affected spontaneous resorption in CBA females mated with syngeneic (CBA) males (PDL1, Fig. 2 B; PDL2 and B7.2, unpublished data).

We then assessed fetal survival rates by allowing plugged females to go to term and counting litter size. Normal litter size was 5.5 pups/plugged female in untreated controls (Ta-
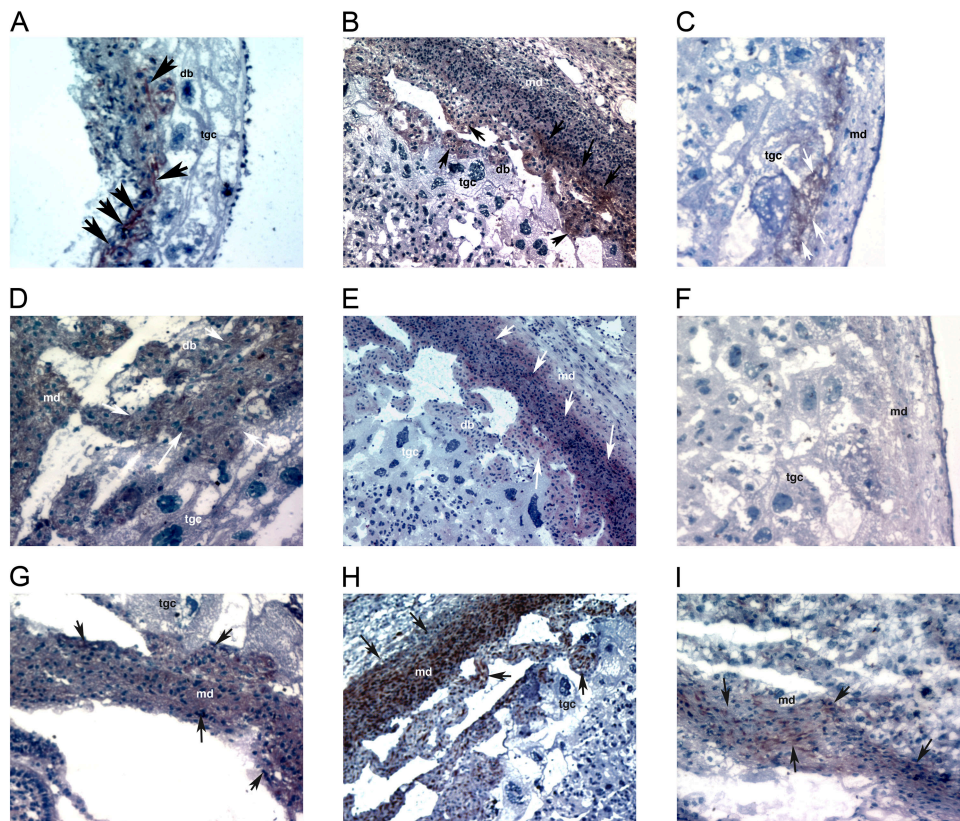

Figure 1. Kinetics of expression of PDL1/PDL2/B7.2 in placenta of CBA mice. Placentas were removed from pregnant CBA (xB6) mice at 10.5, 13.5, and 16.5 dpc, and cryosections were immunostained for PDL1 (A–C), PDL2 (D–F) and B7.2 (G–I) expression. Staining in placentas at 10.5 (A, D, and G), 13.5 (B, E, and H), and 16.5 (C, F, and I) dpc is shown. Reddish-brown staining for PDL1 shown by arrows is in the decidua basalis (db), the layer next to the trophoblastic giant cells (tgc). PDL2 and B7.2 reddish-brown staining depicted by arrows is in the whole decidua (maternal deciduas, md). Magnification is 20 for all sections except section (C), where it is 40 because the staining was faint and difficult to appreciate at 20×.
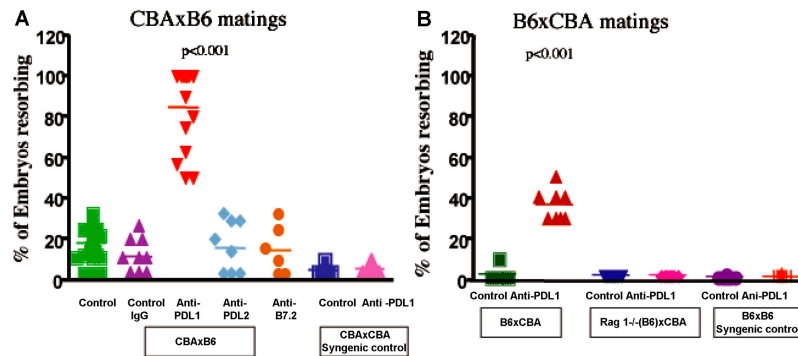
Figure 2.   Effect of PDL1/PDL2/B7.2 blockade on resorption of allogeneic concepti. (A) Pregnant CBA (xB6) females were injected i.p. with anti-PDL1, anti-PDL2, and anti-B7.2 antibodies. The percentages of resorbing fetuses at day 13.5 of pregnancy in mice treated with anti-PDL1 (▼, red), anti-PDL2 (◆, blue), and anti-B7.2 (●, organe). Data in unmanipulated control mice (■, green) and IgG-treated control mice (▲, magenta) are also shown. Data in the anti-PDL1 group are highly significant (P < 0.0001) over other groups. (B) RAG1$^{-/-}$ females on C57BL/6 background were mated with CBA males and subsequently treated with con-

trol IgG or anti-PDL1 antibodies. In parallel, C57BL/6 females were mated with CBA males and subsequently treated with either anti-PDL1 mAb or control IgG. Number of embryos resorbing in each group is shown. (■, green) B6xCBA control; (▲, red) anti-PDL1; (▼, blue) RAG-$^{1-/-}$B6xCBA control; (◆, pink) anti-PDL1. The lack of effect of anti-PDL1 during syngeneic pregnancy for CBA x CBA matings or B6 x B6 matings are shown in A (■, blue, control; ▲, pink, anti-PDL1) and B (●, magenta, control; +, red, anti-PDL1), respectively.

ble I). Anti–PDL1 treatment resulted in significant reduction in the litter size to 1.25 (P < 0.0001 by unpaired *t* test). Treatment with anti-PDL2 or anti-B7.2 mAb did not affect fetal survival rates. As expected, similar numbers of syngeneic (CBA x CBA) fetuses were delivered at term in mice treated with anti-PDL1, and survival rates were comparable with rates observed in control (IgG-treated or unmanipulated) mice (Table I).

Collectively, our data indicate that PDL1 is an important negative regulator of the maternal alloimmune responses against fetal antigens in vivo. In some models, PDL1 has been shown also to interact with a putative receptor other than PD1 to deliver positive stimulatory signals for T cell activation (19–21). Our data clearly show that PDL1 delivers negative signals to protect from allogeneic fetal destruction during pregnancy, because blockade of this pathway resulted in enhanced fetal rejection. However, one of the potential explanations for our findings is that the anti-PDL1 mAb may

act in an agonistic fashion, delivering an activating signal to PDL1 on APCs and/or T cells. Therefore, we used PDL1–deficient mice in an allogeneic mating model. PDL1-deficient animals are on 129/B6 background. We therefore reversed the mating for this set of experiments; 129/B6 females were mated with CBA males and followed to term

Table I. Effect of anti-PDL1 treatment on fetal survival at term

Mating combination	Treatment	Pregnant females	Decidua per female
CBA x CBA	anti-PDL1	8	9.0 ± 0.75
	anti-PDL2	4	8.2 ± 1.25
	anti-B7.2	4	8.2 ± 0.96
	control IgG	4	9.0 ± 0.96
CBA x B6	anti-PDL1	12	1.2 ± 0.87[a]
	anti-PDL2	6	5.8 ± 1.6
	anti-B7.2	7	5.7 ± 1.1
	control IgG	10	5.5 ± 0.97

[a]P < 0.0001 by unpaired Student's *t* test.

Table II. Fetal survival at term in PDL1-deficient mice

Mating combination	Pregnant females	Decidua per female
PDL1$^{-/-}$(129/B6) F x PDL1$^{-/-}$(129/B6) M syngeneic	12	8.0 ± 0.95
PDL1$^{-/-}$(129/B6) F x CBA M allogeneic	9	2.7 ± 1.6[a]
PDL1$^{+/-}$(129/B6) F x PDL1$^{+/-}$(129/B6) M syngeneic	18	8.9 ± 0.7
PDL1$^{+/-}$(129/B6) F x CBA M allogeneic	14	8.5 ± 0.7
PDL1$^{+/+}$(129/B6) F x PDL1$^{+/+}$(129/B6) M syngeneic	5	8.8 ± 0.98
PDL1$^{+/+}$(129/B6) F x CBA M allogeneic	6	9.0 ± 0.8
CBA F x PDL1$^{-/-}$(129/B6) M	4	6.0 ± 0.8
CBA F x PDL1$^{+/-}$(129/B6) M	5	6.0 ± 0.7

[a]P < 0.0001 by unpaired Student's *t* test.

pregnancy. As illustrated in Table II, PDL1-deficient mice had significant reduction in fetal survival rates as assessed by the litter size from the plugged females (2.7 ± 1.6 vs. 8.5 ± 0.7 from the heterozygous and 9 ± 0.8 from WT littermate; P < 0.0001 by unpaired t test for both). Again, fetal resorption was observed in allogeneic and not in syngeneic (B6 x B6) mating, thereby confirming that fetal resorption was in response to male alloantigens.

Our data in PDL1-deficient mice complement our findings of PDL1 blockade with antibody and provide definitive proof that PDL1 delivers a negative signal to regulate allogeneic T cell responses in fetomaternal tolerance. A regulatory role for PDL1 in immune response also is supported by a recent report in which PDL1-deficient mice developed severe experimental autoimmune encephalomyelitis after adoptive transfer of MOG-specific T cells (22).

Role of T cells and complement

We next analyzed the cellular infiltrates at the fetomaternal interface by immunohistochemistry. Infiltration of T cells at the site of fetal rejection was observed as early as 10.5 dpc in anti-PDL1 mAb-treated animals (Fig. 3). We detected negligible infiltration of CD3$^+$ T cells in tissue sections prepared from control IgG-treated mice even though this group has a spontaneous resorption rate of ~18%. This spontaneous resorption rate could be caused by a difference in kinetics of recruitment of T cells in this group. There was no infiltration of T cells in sections from mice that carried syngeneic fetuses and had been treated with anti-PDL1 mAb (unpublished data). There were also sites of hemorrhage during fetal

rejection in the anti-PDL1–treated group as shown by hematoxylin and eosin staining (Fig. 3 C).

We also conducted immunohistological analyses to determine whether innate effector mechanisms involving complement were activated when we blocked the costimulatory pathways in vivo. Staining with antibodies against C3 revealed that complement was deposited at the maternal–fetal interface from early gestational times in mice that were carrying allogeneic concepti and were exposed to anti-PDL1 mAb. C3 deposition was detected as early as 8.5 dpc and was maximal at 10.5 dpc (Fig. 3). C3 staining was concentrated both in the fetal compartment (on trophoblast cells; Fig. 3 D) and in the maternal decidua (Fig. 3 E).

We then mated female RAG-1$^{-/-}$ mice, which lack T cells and B cells but have APCs (on C57BL/6 background), with CBA males and treated them with anti-PDL1 mAb. In parallel sets of experiments, C57BL/6 females (RAG sufficient) were mated with CBA males and were subsequently treated with anti-PDL1. All RAG-1$^{-/-}$ females examined at 13.5 dpc in the anti-PDL1–treated group had normal numbers of healthy embryos (Fig. 2 B), whereas in C57BL/6 (RAG sufficient) females anti-PDL1 treatment resulted in 35% fetal resorption (two-tailed P < 0.0001 by unpaired t test). A lack of effect of anti-PDL1 mAb in RAG-deficient females would suggest that maternal T cells and B cells are essential for rejection of allogeneic concepti to occur in mice treated with anti-PDL1 mAb. Because RAG-1$^{-/-}$ mice also lack B cells, we used B cell–deficient mice (that have T cells and APCs intact) for allogeneic mating. Anti-PDL1 mAb caused fetal rejection in four of four B cell–deficient mice;
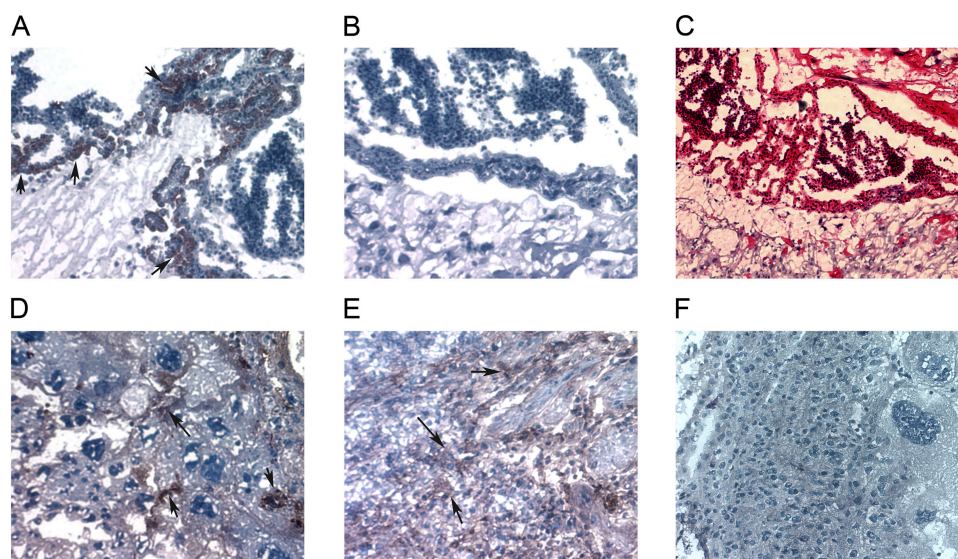

Figure 3. T cell infiltration and complement deposition in placentas of anti-PDL1–treated CBA females. CBA (xB6) females were treated with anti-PDL1 at predetermined intervals. Animals were killed, and placental sections were stained for T cells (A, B) and complement (D–F). A depicts T cells stained with anti-CD3 mAb (reddish-brown staining as shown by arrows), and C (whole area depicted) shows a site of hemorrhage during fetal rejection in anti-PDL1–treated group. Arrows show complement deposition in the fetal (D) and maternal (E) part of the placenta. Lack of staining by isotype control is shown in B for T cells and in F for complement. Magnification is 20 for all the sections except C, where it is 10 (to show the whole area undergoing hemorrhage).

the percentage of resorbed embryos was 35–40%, similar to that obtained in the WT C57BL/6 x CBA mating combination with anti-PDL1 mAb treatment. Again, in syngeneic (B6 x B6) mating, anti-PDL1 treatment did not cause resorption (Fig. 2 B). Our results clearly show that T cells and not B cells or APCs are required for anti-PDL1–mediated fetal rejection.

Role of IDO in anti-PDL1–mediated fetal rejection

Inhibition of IDO recently was shown to result in fetal rejection in the CBA x B6 allogeneic pregnancy model (5, 6). To test whether anti-PDL1 mAb may function by enhancing IDO expression, as has been reported for CTLA4Ig (23), we investigated the expression of IDO at the maternal–fetal interface by immunohistochemistry. A similar degree of IDO expression was detected in the trophoblastic giant cells of placenta from control and anti-PDL1 treated mice (unpublished data), indicating that anti-PDL1 treatment has no effect on IDO expression.

Effect of PDL1 on Th1 cells

We then studied the peripheral immune response of anti-PDL1–treated mice. Splenocytes (responder cells) from pregnant CBA females were cultured in the presence of irradiated stimulator cells from CBA or B6 males. Using ELISPOT analysis, we detected a high frequency of IFN-γ– producing cells in response to stimulator cells from B6 (allogeneic) males as opposed to CBA males with anti-PDL1 mAb treatment (Fig. 4 A), indicating that there is expansion of alloreactive Th1 cells in vivo. Th2 cytokines were not de-

tected in either control or anti-PDL1 treated mice. In a parallel study, splenocytes (responder cells) from pregnant PDL1-deficient mice (on 129/B6 background) were cultured in the presence of irradiated stimulator cells from CBA (allogeneic) mice. ELISPOT studies again revealed high frequency of IFN-γ–producing alloreactive cells in PDL1-deficient mice (Fig. 4 B). Splenocytes from WT littermate controls (129 x B6) had minimal IFN-γ–producing cells in response to allogeneic (CBA) stimulators (Fig. 4 B).

We next examined the expression of IFN-γ locally at the site of fetal rejection in the placenta by analysis of placental homogenates by ELISA (24). We found higher levels of IFN-γ in placental homogenates from anti-PDL1–treated mice (Fig. 4 C) and from PDL1-deficient mice as compared with appropriate controls (Fig. 4 D).

The balance of Th1/Th2 cytokines has been suggested to be crucial for outcome of a healthy pregnancy (25, 26). Therefore, expansion of Th1 effector cells by anti-PDL1 mAb treatment and in PDL1-deficient mice could be a major contributing factor to enhanced fetal rejection in our model.

In conclusion, our data provide definitive proof for a critical role of PDL1 in promoting fetomaternal tolerance. The data show that blocking or genetically deleting this costimulatory molecule promotes fetal rejection by expansion of alloreactive Th1 cells. Thus, PDL1 may contribute to fetomaternal tolerance by limiting the expansion of alloreactive T cells (13, 27), possibly by cell cycle arrest (28), by increasing apoptosis of T cells (13, 29, 30), or by active regulation of the alloimmune response by a subpopulation of $CD4^+CD25^+$ T cells (13, 31). Further studies are necessary
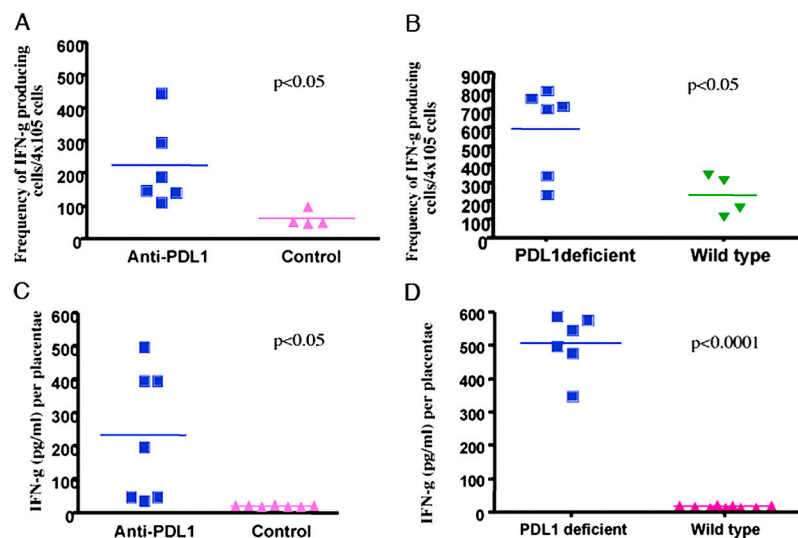

Figure 4.   Expansion of Th1 cells in spleen and placenta of anti-PDL1–treated and PDL1-deficient mice. (A) The frequency of IFN-γ–producing cells from splenocytes of anti-PDL1–treated animals was measured by ELISPOT from a mixed leukocyte reaction in which splenocytes (responder cells) from pregnant CBA mice were cultured in the presence of allogeneic (C57BL/6) stimulators ($n = 4$–6; $P < 0.05$). (B) The frequency of IFN-γ–producing cells from splenocytes of PDL1-deficient mice was mea-

sured by ELISPOT in a similar fashion ($n = 4$–6 mice per group; $P < 0.05$ as compared with PDL1$^{+/+}$ WT littermate controls). (C) IFN-γ was measured in the placental homogenates by ELISA from anti-PDL1–treated mice ($n = 7$; $P < 0.05$ compared with control group). (D) IFN-γ in placental homogenates of PDL1-deficient mice is also shown ($n = 6$–9 mice per group; $P < 0.0001$ compared with WT PDL1$^{+/+}$ littermates).

to define the exact mechanisms of the interplay between PDL1, effector T cells, and regulatory T cells in vivo. Our studies have important implications for understanding physiologic mechanisms that promote fetomaternal and transplantation tolerance.

MATERIAL AND METHODS

Mice. CBA/CaJ, C57BL/6, RAG-1$^{-/-}$, and B-deficient mice on C57BL/6 background were obtained from Jackson ImmunoResearch Laboratories. Institutional guidelines for animal care and experimental procedures were followed.

Timed matings and resorption rates. Virgin female CBA/CaJ mice (aged 8–10 wk) were mated with C57BL/6 (allogeneic) or CBA/CaJ (syngeneic) males (aged 6–12 wk). Females were inspected daily for vaginal plugs; sighting a vaginal plug was designated as d 0.5 of pregnancy. Plugged females were monitored until parturition, and the number of pups born was recorded, or mice were killed at predetermined intervals (10.5, 13.5, 16.5 dpc) to examine the number of implantation and resorbing sites. The rate of resorption was calculated by counting the number of resorbing versus healthy embryos on d 13.5. RAG-1$^{-/-}$ and B cell–deficient mice on C57BL/6 background were mated with CBA males to set up allogeneic matings. B6 x B6 matings served as syngeneic controls in this group.

Treatment protocol. Pregnant females were injected i.p. 6.5, 8.5, 10.5, and 12.5 dpc with the blocking anti–mouse PDL1 mAb (MIH6) at dosages of 500 μg, 500 μg, 250 μg, and 250 μg, respectively. In parallel sets of experiments, groups of mice were injected with anti-PDL2 (TY25) (32) or anti–B7.2 (GL1) (33) antibodies with the same dosages and regimen.

Histology. At predetermined intervals, placentas were removed for histological analysis and immunostaining. Placentas were embedded in Tissue Tek OCT (Sakura Finetek) compound and frozen in liquid nitrogen. Hematoxylin and eosin staining was done at different time-point placentas. Immunohistochemistry was performed on frozen tissue sections with antibodies to PD1 (J 43), PDL1 (MIH6), PDL2 (TY25), B7.1 (1G10), B7.2 (2D10), CTLA4 (4F10), CD3 (145-2C11) and complement (CI: 11H9) using avidin-biotin technique (Vector Laboratories). For cryosections, staining for IDO was adapted from Baban et al. (34).

Detection of cytokines in placental homogenates. Placentas from anti-PDL1 and control groups were taken 13.5 dpc and homogenized in 1 ml PBS with 1% Triton X-100 or PBS alone, a technique described earlier by our group (24). The homogenate was analyzed for IFN-γ, IL-4, IL-5, and IL-10 using matched antibody pairs (BD Biosciences) by ELISA. Cytokines were analyzed in placentas from PDL1-deficient and WT littermate controls in a similar manner.

ELISPOT analysis. Splenocytes from CBA (xB6) pregnant mice, which had been treated with anti-PDL1 or not treated, were obtained as single-cell suspensions and used as responder cells. Irradiated (3,000 rad) splenocytes from male C57BL/6 and CBA/CaJ mice were used as stimulator cells. The ELISPOT assay was adapted to measure IFN-γ– and IL-4–secreting cells, as previously described (27, 35, 36). In another set of experiments, splenocytes from PDL1-deficient mice were used as responder cells to perform similar ELISPOT analysis. Splenocytes from pregnant PDL1-deficient mice (on 129/B6 background) (xCBA) were used as responder cells, and splenocytes from CBA males were used as stimulators.

Generation of PDL1–deficient mice. To confirm the role of PDL1 in fetomaternal tolerance, we generated PDL1-deficient mice. A targeting vector was constructed using an 8-kb genomic fragment containing exons 2 and 3 of the PDL-1 gene (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20050019/DC1). A 0.8-kb sequence involving exons 2 and 3 was deleted and replaced by pMC-1 neo, the targeting vector was linearized

and electroporated into embryonic stem cells, and the PDL-1$^{+/-}$ embryonic stem cell clones were selected in media containing G418 and ganciclovir. Correct targeting was assessed by Southern blotting (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20050019/DC1), and chimeric mice were derived by blastocyst injection. Offspring were screened by PCR using primers 5′-GAATGAACAAACGAGCGAGAA-3′ and 5′-GCAGC-GCATCGCCTTCTATC-3′ to detect KO allele and primers 5′-TCACG-GCTCCAAAGGACTTGT-3′ and 5′-CCGCACCACCGTAGCTG-3′ to detect WT allele. PDL1 mice were backcrossed to the C57/BL6 strain and maintained in our facility for experimental use.

Online supplemental material. Fig. S1 shows the genomic organization of the PDL1 gene and the structure of the PDL1 targeting vector. Fig. S2 shows the deletion of PDL1 exons 2 and 3 in the KO allele by Southern blot. Genomic digestion with EcoRI yields 11kb fragment in the WT allele and a 7-kb fragment in the KO allele. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20050019/DC1.

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