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Original Paper

Human placenta-derived stromal cells decrease inflammation, placental injury and blood pressure in hypertensive pregnant mice

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Abstract

Pre-eclampsia, the development of hypertension and proteinuria or end-organ damage during pregnancy, is a leading cause of both maternal and fetal morbidity and mortality, and there are no effective clinical treatments for pre-eclampsia aside from delivery. The development of pre-eclampsia is characterized by maladaptation of the maternal immune system, excessive inflammation and endothelial dysfunction. We have reported that detection of extracellular RNA by the Toll-like receptors (TLRs) 3 and 7 is a key initiating signal that contributes to the development of pre-eclampsia. PLacental eXpanded (PLX-PAD) cells are human placenta-derived, mesenchymal-like, adherent stromal cells that have anti-inflammatory, proangiogenic, cytoprotective and regenerative properties, secondary to paracrine secretion of various molecules in response to environmental stimulation. We hypothesized that PLX-PAD cells would reduce the associated inflammation and tissue damage and lower blood pressure in mice with pre-eclampsia induced by TLR3 or TLR7 activation. Injection of PLX-PAD cells on gestational day 14 significantly decreased systolic blood pressure by day 17 in TLR3-induced and TLR7-induced hypertensive mice (TLR3 144–111 mmHg; TLR7 145–106 mmHg; both $P < 0.05$), and also normalized their elevated urinary protein:creatinine ratios (TLR3 5.68–3.72; TLR7 5.57–3.84; both $P < 0.05$). On gestational day 17, aortic endothelium-dependent relaxation responses improved significantly in TLR3-induced and TLR7-induced hypertensive mice that received PLX-PAD cells on gestational day 14 (TLR3 35–65%; TLR7 37–63%; both $P < 0.05$). In addition, markers of systemic inflammation and placental injury, increased markedly in both groups of TLR-induced hypertensive mice, were reduced by PLX-PAD cells. Importantly, PLX-PAD cell therapy had no effects on these measures in pregnant control mice or on the fetuses. These data demonstrate that PLX-PAD cell therapy can safely reverse pre-eclampsia-like features during pregnancy and have a potential therapeutic role in pre-eclampsia treatment.

Key words: hypertension, immunity, pre-eclampsia, Toll-like receptors, vascular diseases.

INTRODUCTION

Pre-eclampsia (PE), the new development of hypertension and proteinuria or end-organ damage during pregnancy, affects approximately 6–8% of pregnancies and its incidence is rising. PE is a major cause of maternal and fetal death and is also associated with increased risk of future cardiovascular disease in both the mother and the infant [1–4]. Physiologically, PE is associated with excessive inflammation, endothelial dysfunction, renal dysfunction and placental injury.

The aetiology of PE remains elusive, but is probably multifactorial and differs between women. There is evidence to support the hypothesis that pre-existing endothelial dysfunction due to obesity, diabetes and/or ageing, combined with the stress of pregnancy, is enough to induce PE [5]. Other evidence supports the theory that inadequate trophoblast invasion, placental

Abbreviations: ACh, acetylcholine; H&E, haematoxylin and eosin; hGM-CSF, human granulocyte–macrophage colony-stimulating factor; HIF1α, hypoxia-inducible factor 1α; hMMP, human matrix metalloprotease; hTIMP, human tissue inhibitor of metalloprotease; IL, interleukin; mEGF, mouse epidermal growth factor; mVEGF, mouse vascular endothelial growth factor; NK, natural killer; PE, pre-eclampsia; P, PBS vehicle; PLA, PlasmaLyte A; PLX-PAD, PLacental eXpanded; PPIC, poly(I:C); PR, R837; SBP, systolic blood pressure; SNP, sodium nitroprusside; TCR, T-cell receptor; TLR, Toll-like receptor.

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ischaemia and angiogenic imbalance result in endothelial dysfunction, leading to PE [6,7]. There are also strong associations between pathogenic infections and the development of PE [8,9]. In addition, there have been reports that women with abnormal immune responses to endogenous (autoimmunity) and exogenous (paternally derived) antigens are highly vulnerable to developing PE during the physiological stress of pregnancy [10,11]. Although all four of these scenarios originate from different initiators, they all involve the innate immune system [12].

Numerous studies in women with PE report excessive immunity and inflammation as well as widespread endothelial dysfunction. The innate immune system responds to both foreign pathogens and endogenous markers of cellular damage [RNA, DNA, heat shock proteins, uric acid, tumour necrosis factor (TNF), etc.] by inducing inflammation via nuclear factor-κB (NF-κB) and interferons. We and others have reported that activation of Toll-like receptors (TLRs), a family of innate pattern recognition receptors, induces pregnancy-dependent hypertension, renal dysfunction, endothelial dysfunction and placental injury [13–18].

There are currently no effective treatments for women diagnosed with PE. We hypothesized that a cell therapy, using specialized cells that produce and secrete factors capable of suppressing inflammation and protecting cells from injury, may be beneficial in treating PE. PLacental eXpanded cells (PLX-PAD cells; Pluristem Therapeutics, Inc.) are human placenta-derived, mesenchymal-like, adherent stromal cells that have anti-inflammatory, proangiogenic, cytoprotective and regenerative properties, secondary to paracrine secretion of various molecules in response to environmental stimulation. PLX-PAD cells have been reported to display therapeutic potential in a variety of studies including amelioration of hindlimb ischaemia and reduction of heart tissue damage after myocardial infarction [19,20]. In addition, they improved cardiac function in mice with streptozotocin-induced cardiomyopathy, regenerated damaged skeletal muscle in both murine and phase 2 clinical studies, reduced pulmonary fibrosis and ameliorated experimental autoimmune encephalomyelitis (unpublished data). PLX-PAD cells are being tested in numerous clinical trials for peripheral arterial disease, muscle injury and pulmonary hypertension, but whether they might be beneficial for treating PE is unknown. We hypothesized that PLX-PAD cells would reduce the inflammation, and endothelial, renal and placental injury and dysfunction, and lower blood pressure in mice made pre-eclamptic by TLR activation.

METHODS

Animals and treatments

Female and male C57BL/6J mice were purchased from Jackson Laboratories. All procedures were approved by the Texas A&M Health Science Center/Baylor Scott and White Health IACUC (Institutional Animal Care and Use Committee) in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*. After mating, all pregnant mice aged 10–12 weeks were given intraperitoneal injections, on days 13, 15 and 17, of vehicle (PBS or 'P'), the TLR3 agonist poly(I:C) ('PPIC'; 20 mg/kg) or the TLR7 agonist R837 ('PR'; 4 mg/kg) as described previously [14,15]. On day 14, the P, PPIC and PR mice were given two 25-μl intramuscular injections, into the right leg, of either vehicle (PlasmaLyte A or 'PLA') or PLX-PAD cells (40000, 200000 or 1×10^6 total cells; 'PLX'). The detailed characteristics, including cell surface expression markers of PLX-PAD cells, have been described previously [19,20]. Selected groups of P and PPIC mice received 1×10^6 cells of either human adipose cells or bone marrow cells on day 14, as above. Pluristem isolated the human adipose cells from a liposuction aspirate and bone marrow cells were purchased (PromoCell).

Cardiovascular, renal and fetal development measures

Tail-cuff systolic blood pressures were measured (IITC, Inc.) at baseline and daily between days 13 and 17, before the injections, as described previously [14]. Mice were weighed and then euthanized on day 18 of pregnancy, and blood, urine and tissues were collected. Urinary protein and creatinine concentrations, aortic relaxation responses, and number of pups and fetal demise were determined as described previously [14,15]. Briefly, commercially available ELISA kits were used to quantify urinary protein and creatinine concentrations, and the ratio was calculated. Endothelium-intact aortic segments (2 mm), gently cleaned of adipose and connective tissue, were mounted on pins in a myograph to measure isometric force generation. After acclimatization and verification of the integrity of the endothelium, vessels were contracted with phenylephrine, followed by increasing concentrations of acetylcholine (ACh). After the final concentration had been added, the chambers were rinsed out and the vessels relaxed to baseline. Then phenylephrine was again added, followed by increasing concentrations of sodium nitroprusside (SNP) to measure endothelium-independent relaxation responses.

Inflammation measures

Spleens from all mice were isolated and weighed and then processed for flow cytometry, as described previously [14,15]. Splenocytes were stained for $\gamma\delta$ T-cells using an anti-$\gamma\delta$ T-cell receptor (TCR)-FITC antibody (eBioSciences), and gating was performed based on anti-$\gamma\delta$ TCR-allophycocyanin(APC) isotype controls (eBioSciences). Whole blood was processed, and ELISAs to quantitate plasma interleukin (IL)-6 and IL-4 levels were performed according to the manufacturer's protocol (R&D Systems).

Placental measures

Placentas were isolated, protein was extracted and immunoblotting was performed on some samples to quantify hypoxia-inducible factor 1α (HIF1α) levels, whereas the other placentas were paraffin embedded and processed for histological studies using haematoxylin and eosin (H&E) staining to assess necrosis as previously described [14,15]. Other placentas were used for immunofluorescence studies to identify the amount of annexin V (FITC) staining for apoptosis (In Situ Cell Death Detection Kit-Fluorescein, Roche). DAPI was used to identify cell nuclei. Images were obtained using an Olympus Bx51 microscope equipped with an Olympus DP72 camera and 10×, 20× and 40× UPLANFL-N objectives with numerical apertures of α-FN 26.5,

0.17-FN 26.5 and 0.17-FN 26.5, respectively. The acquisition software was Cell Sens, and Image J was used for subsequent processing of images. For quantification of necrotic areas in the placenta, Image J was used to measure the total area of the image followed by the necrotic areas; the necrotic areas were expressed as a percentage of the total area. For quantification of apoptotic cells, again Image J was used to count the DAPI-stained nuclei in each image followed by the number of annexin V-positive cells. These data on apoptotic cells were expressed as a percentage of the total cells.

Growth factor measures

Luminex studies were performed using plasma obtained on days 15 and 18 to identify mouse and human proteins that may be induced or secreted by PLX-PAD cells. For day 15 analyses, the Milliplex Magpix System (Millipore) was used, together with the Luminex panels LXSAHM-14 (human 14-plex assay, R&D Systems) and MAGPMAG-24K-06 (Mouse Angiogenesis/Growth Factor Panel, Millipore). Data were analysed using the Milliplex Analyst software (Merck Millipore). For day 18 analyses, Luminex studies were performed in mouse plasma to identify human proteins that may be induced or secreted by PLX-PAD cells. The Milliplex MAP Human Cytokine/Chemokine Magnetic Bead Panel – Premixed 38 Plex – Immunology Multiplex Assay (HCYTMAG-60K-PX38, Millipore) was used following the manufacturer's protocol. The plate was incubated overnight at 4 °C and washed using the hand-held magnet technique. The plate was run on the Bio-Plex 200 system (BioRad) and the data were analysed using the Bio-Plex SNP Manager software (BioRad). The minimum criterion was defined as 100 events per bead region within the device settings.

Statistical analyses

Results are presented as means $\pm$/+ S.E.M.s. A one-way analysis of variance was used followed by Student's Newman–Keuls post-hoc test when necessary. Temporal changes of systolic blood pressure (SBP) in mice were analysed using a repeated-measures analysis of variance, followed by Student's Newman–Keuls post-hoc test when necessary. Circulating human and mouse factors were analysed between groups as well as between PLA and PLX, using a Mann–Whitney rank sum test due to lack of normality. The significance level was 0.05.

RESULTS

We first examined whether PLX-PAD cell therapy could decrease the pregnancy-dependent hypertension induced by TLR3 or TLR7 activation. Pregnant mice were treated with vehicle (P), PPIC or PR, given by intraperitoneal injection on gestational days 13, 15 and 17, while also receiving two intramuscular (right leg) injections of either PLX-PAD cells (1×10^6 total) or vehicle (PLA) on gestational day 14 only. SBP was measured at baseline and daily from gestational day 13 to gestational day 17, before any injections. TLR activation significantly increased SBP compared with vehicle-treated pregnant controls (gestational day 17: P+PLA 100$\pm$4 mmHg, PPIC+PLA 144$\pm$3 mmHg, PR+PLA 145$\pm$2 mmHg; all $P < 0.05$ vs P+PLA) (Figure 1a). PLX-PAD cell treatment progressively normalized SBP in PPIC and PR mice while having no effect in P mice (Figure 1a).

We next tested whether this effect was dose dependent. In PPIC mice only, we injected either PLA or PLX-PAD cells at 1×10^6, 200000 or 40000 cells on gestational day 14 as above. There was a significant concentration-dependent decrease in SBP in the PPIC mice (Figure 1b). In addition, we determined whether the antihypertensive effects of PLX-PAD cells were cell source specific or whether other stromal cells could have the same effect. We injected 1×10^6 human adipose cells or bone marrow cells, known to have low immunogenicity, into the right leg muscle of P and PPIC mice, as above. In contrast to mice that received PLX-PAD cells, PPIC mice receiving human adipose or bone marrow cells did not exhibit a decrease in SBP by gestational day 16 (Figure 1c). These data demonstrate that human PLX-PAD cells, but not stromal cells from human adipose or bone marrow sources, can ameliorate the hypertension caused by TLR activation during pregnancy.

We then examined whether TLR3 or TLR7 activation with PPIC or PR caused a significant increase in urinary protein excretion, and whether PLX-PAD cells could prevent this. The ratios of [urinary protein]:[urinary creatinine] were increased significantly in PPIC+PLA and PR+PLA mice compared with P+PLA mice (gestational day 18: P+PLA 3.73$\pm$0.15, PPIC+PLA 5.68$\pm$0.17, PR+PLA 5.57$\pm$0.29; all $P < 0.05$ vs P+PLA) (Figure 2a). PLX-PAD cell treatment prevented the increase in the urinary protein:creatinine ratio on gestational day 18 in PPIC and PR mice, while having no effect in P mice (Figure 2a). In PPIC+PLA mice, neither adipose nor bone marrow cell treatment decreased urinary protein:creatinine ratios (5.73$\pm$0.25 and 5.42$\pm$0.29, respectively).

We also examined whether PLX-PAD cell treatment had any detrimental effects on fetal development. There were no differences in the number of pups/litter (range of means among groups = 7.5–8.5 pups), in the incidence of fetal demise in all groups (range of means among groups = 0.125–0.75 pups), or in total litter weight determined by weighing the intact uterine horn containing pups, placentas and amniotic fluid (range of means among groups = 6–8 g) (Figure 2b and not shown) in any of the groups. We also tested whether pups exposed to PLX-PAD cells might have reproductive difficulties once they reached adulthood. Male and female offspring from P, PPIC and PR groups that received PLA or PLX-PAD cells were mated with control mice aged between 9 and 10 weeks, and the ability to produce a litter of live pups was analysed. PLX-PAD cell treatment had no effect on the ability to produce and develop offspring (data not shown). In addition, SBP during pregnancy was normal in both female offspring exposed *in utero* to PLX-PAD cells and control female mice mated with male offspring exposed *in utero* to PLX-PAD cells (data not shown). Together, these data demonstrate that PLX-PAD cell treatment did not detrimentally affect the fetuses, and independent reproductive toxicology studies performed by Charles River confirmed these findings.

Next we examined whether PLX-PAD cells could reduce the endothelial dysfunction evident in PPIC and PR mice. We confirmed that *ex vivo* aortic relaxation responses to the

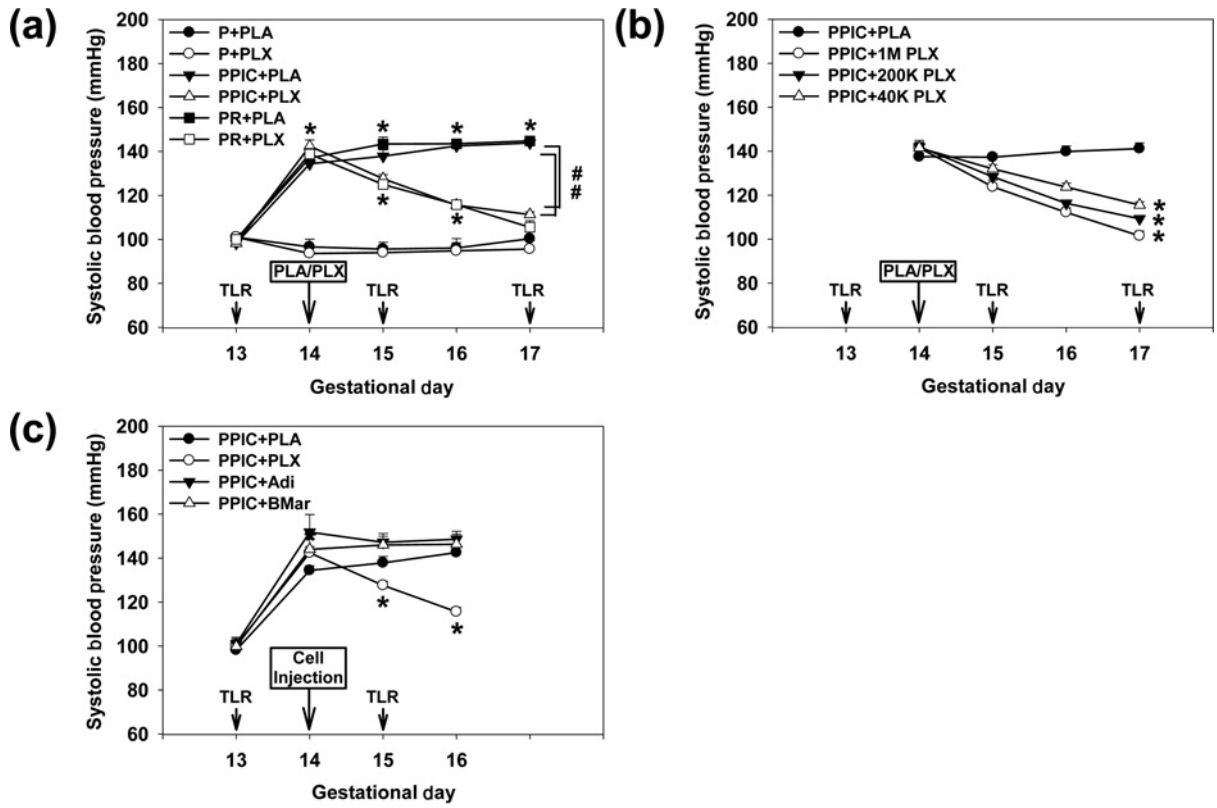

Figure 1 PLX-PAD cells decreased the hypertension caused by TLR3 and TLR7 activation in pregnant mice

(**a**) Pregnant mice either untreated (P), or treated with PPIC or PR on days 13, 15 and 17, developed hypertension, which was prevented by injection of 1×10^6 PLX-PAD cells (PLX) on day 14 of the pregnancy, whereas the vehicle PLA had no effect. $^*P<0.05$ vs P, $^\#P<0.05$ vs PLA of the same group. (**b**) Injection of 1×10^6, 200000 or 40000 PLX-PAD cells significantly decreased SBP in a dose-dependent manner. $^*P<0.05$ vs PPIC+PLA. (**c**) PLX-PAD cells (1×10^6) decreased SBP in PPIC mice; however, 1×10^6 human adipose (Adi) or bone marrow (BMar) cells had no effect. Results are expressed as means+S.E.M.s; $n = 6$–8 mice in each group.

endothelium-dependent dilator ACh were decreased significantly in PPIC+PLA and PR+PLA mice (Figure 2c), but responses to the endothelium-independent dilator SNP were not altered (Figure 2d). PLX-PAD cell treatment normalized aortic ACh-induced relaxation responses in PPIC and PR mice while having no effects in P mice or on SNP-induced relaxation responses (Figures 2c and 2d).

Using our immune system-mediated model of PE, we determined what effects PLX-PAD cell treatment had on immune system activation and inflammation in these mice. Neither TLR activation during pregnancy nor PLA/PLX-PAD cell treatment affected body weight because all groups averaged 30–32 g on gestational day 18. PPIC+PLA and PR+PLA mice exhibited splenomegaly at gestational day 18, and PLX-PAD cell treatment significantly attenuated the increase in spleen weight:body weight ratios (Figure 3a). In contrast, adipose or bone marrow cell treatment had no effect on spleen weight:body weight ratios in PPIC+PLA mice (5.8 ± 1.27 and 5.24 ± 0.73, respectively). Significantly increased splenic levels of $\gamma\delta$ TCR+ T-cells were also observed in PPIC+PLA and PR+PLA mice compared with P mice; this parameter was also largely or completely normalized in PLX-treated mice (Figure 3b). To assess the overall proinflammatory/anti-inflammatory status of the mice, we measured plasma levels of the proinflammatory cytokine IL-6 as well as the anti-inflammatory cytokine IL-4 on gestational day 18. Both PPIC+PLA and PR+PLA mice had significantly increased levels of IL-6 (Figure 3c) and significantly decreased levels of IL-4 (Figure 3d), and these levels were restored to normal by PLX-PAD cell treatment (Figures 3c and 3d). These data demonstrate that PLX-PAD cell treatment was able to reduce the splenomegaly, immune system activation and excessive inflammation induced by TLR activation in pregnant mice.

As the placenta plays a role in the development of PE, we examined how it is altered by TLR activation and whether these changes could be prevented by PLX-PAD cell treatment. Placental protein levels of HIF1α, which is known to be induced by TLR activation and mediates hypoxia-induced tissue damage [21], were significantly increased in PPIC+PLA and PR+PLA mice but were reduced in PPIC+PLX and PR+PLX mice, compared with P mice (Figure 4a). We also determined the extent of placental necrosis and apoptosis by H&E and annexin V staining, respectively. There were no signs of placental necrosis in P mice that received either PLA or PLX-PAD cells (Figure 4b). In contrast, there were significant areas of necrosis in PPIC+PLA and PR+PLA placentas (representative images in Figures 4c and 4d, quantification in Figure 4e), whereas placentas from PPIC+PLX

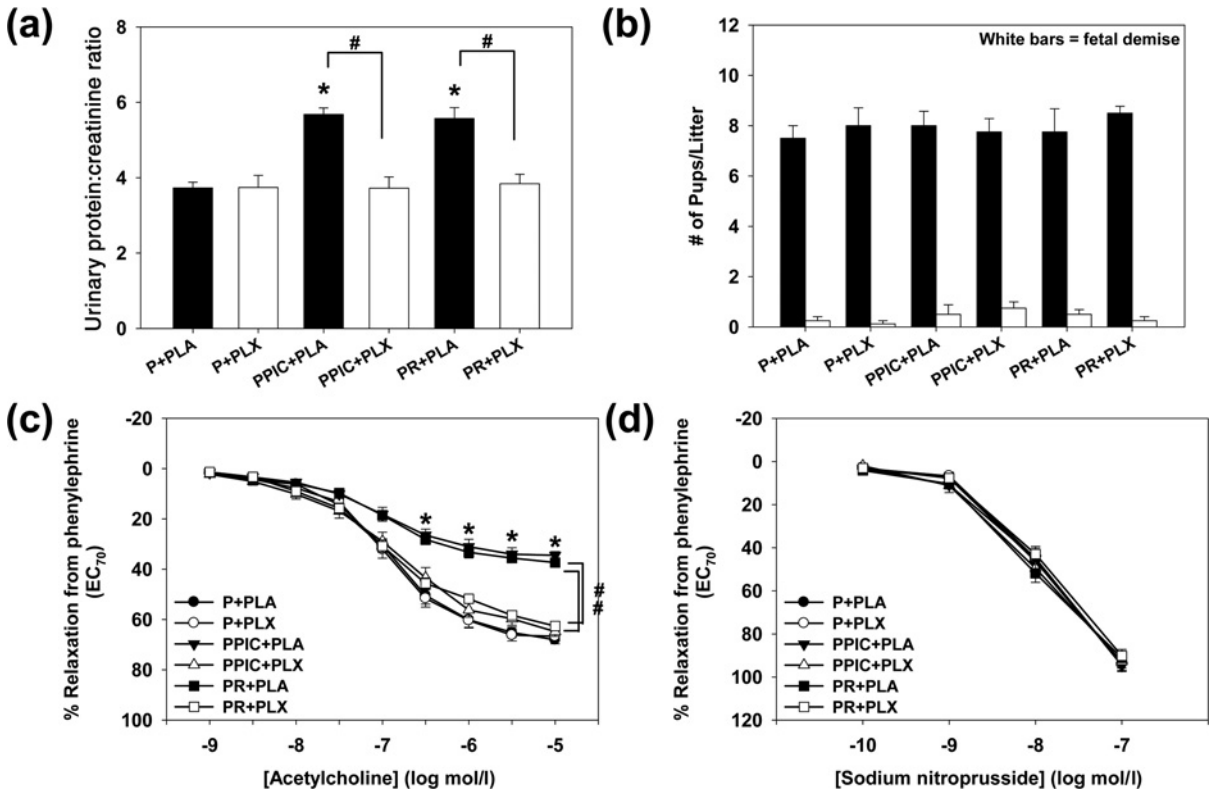

Figure 2 PLX-PAD cells reduced PE-like features in mice

(**a**) Pregnant (P) mice treated with PPIC or PR on days 13, 15 and 17 had significantly increased ratios of urinary protein:albumin, which were normalized by injection of 1×10^6 PLX-PAD cells (PLX) on day 14 of pregnancy, whereas the vehicle PLA had no effect. *$P < 0.05$ vs P, #$P < 0.05$ vs PLA of the same group. (**b**) PLX-PAD cell therapy had no effect on either the number of pups per litter (black bars) or the number of pups exhibiting fetal demise (white bars). (**c**) PLX-PAD cells (1×10^6) significantly increased aortic relaxation responses to ACh in PPIC and PR mice. *$P < 0.05$ vs P, #$P < 0.05$ vs PLA of the same group. (**d**) There were no differences in aortic relaxation responses to the nitric oxide donor SNP in any of the groups. Results are expressed as means+/$\pm$S.E.M.s; $n = 6$–8 mice in each group.

and PR+PLX mice did not exhibit any areas of necrosis (Figures 4c–4e). Higher magnification images of the areas of placental necrosis are provided in Supplementary Figure S1. With respect to apoptosis, there was some annexin V staining in control P mice, and this was reduced modestly by PLX-PAD cell treatment (see Supplementary Figure 2a). Annexin V staining was increased significantly in PPIC and PR placentas compared with P placentas, and this increase was also attenuated by PLX-PAD cell treatment (see Supplementary Figures S2b–S2d). We also examined placental vascular injury using H&E staining and found areas of eosin-positive cells in both the decidua and spongiotrophoblasts of both PPIC+PLA and PR+PLA mice, which were reduced by treatment with PLX-PAD cells (see Supplementary Figure S3). These data support the hypothesis that intraperitoneal injections of TLR agonists can induce immunity and inflammation, which negatively affect placental structure, and intramuscular injections of PLX-PAD cells during pregnancy can safely restore placental health by reducing cell death and tissue injury.

To identify potential factors that might be involved in the anti-PE effects of PLX-PAD cells, we isolated plasma samples from PLA-treated and PLX-PAD cell-treated control, PPIC and PR mice 24 h after PLA or PLX-PAD cell treatment, and analysed them with Luminex. As depicted in Figures 5a and 5b, a significant induction of human matrix metalloprotease 1 (hMMP-1) and human tissue inhibitor of metalloprotease 1 (hTIMP-1) was observed in mice injected with PLX-PAD cells compared with PLA ($P = 0.0001$ and 0.0004, respectively, PLX-PAD vs PLA). In general, plasma from PLA-treated mice did not contain these proteins, regardless of PE induction, whereas plasma from PLX-treated mice was positive for these proteins, with the highest levels seen in PR mice. A similar pattern was observed for hMMP-2 and hMMP-3 (data not shown). It is interesting that, although levels varied somewhat between the individual animals in each group, the animals that had high levels of hMMP-1 tended also to have high levels of hTIMP-1. Similarly, levels of mouse epidermal growth factor (mEGF) and mouse vascular endothelial growth factor (mVEGF)-A were increased 24 h after PLX-PAD cell administration (Figures 5c and 5d); however, the level of statistical significance was somewhat higher for mEGF than for mVEGF-A ($P = 0.0532$ and 0.0042, respectively, PLX-PAD vs PLA). There was no statistically significant effect of PE induction (PPIC, PR vs P) on mVEGF-A and mEGF and, as with hMMP-1 and hTIMP-1, the animals that had high levels of mVEGF-A

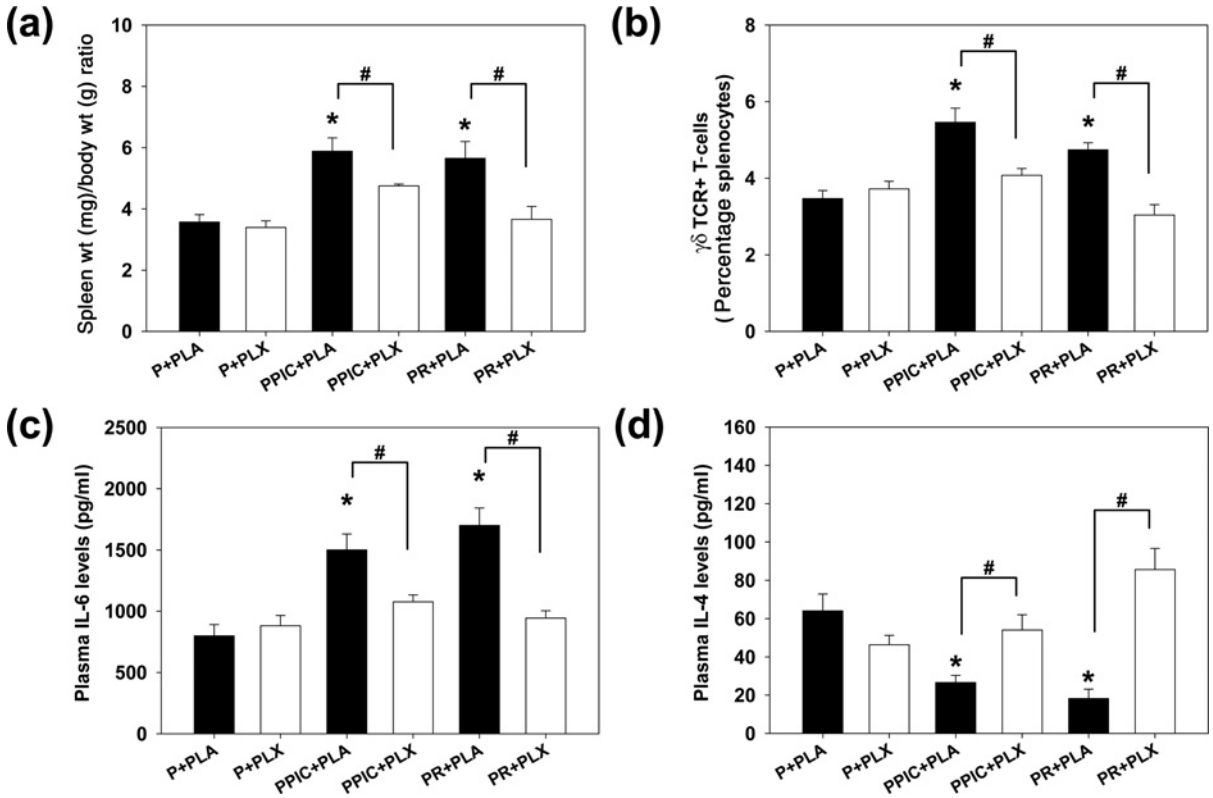

Figure 3 **PLX-PAD cells reduced systemic immunity and inflammation in PE mice**

(**a**) Pregnant mice either untreated (P) or treated with PPIC or PR on days 13, 15 and 17 had significantly increased spleen weight:body weight ratios, which were normalized by injection of 1×10 million PLX-PAD cells (PLX) on day 14 of pregnancy, whereas the vehicle PLA had no effect. $^*P < 0.05$ vs P, $\#P < 0.05$ vs PLA of the same group. (**b**) PPIC and PR mice had significantly increased splenic levels of $\gamma\delta$ TCR+ T-cells, which were decreased by PLX-PAD cells administered on day 14 of pregnancy. $^*P < 0.05$ vs P, $\#P < 0.05$ vs PLA of the same group. (**c**) PPIC and PR mice had significantly increased plasma levels of IL-6, which were decreased by PLX-PAD cells administered on day 14 of pregnancy. $^*P < 0.05$ vs P, $\#P < 0.05$ vs PLA of the same group. (**d**) PPIC and PR mice had significantly decreased plasma levels of IL-4, which were increased by PLX-PAD cells administered on day 14 of pregnancy. $^*P < 0.05$ vs P, $\#P < 0.05$ vs PLA of the same group. Results are expressed as means+S.E.M.s; $n = 6$–8 mice in each group.

were those animals found to have high levels of mEGF. On day 18, plasma from PLX-PAD cell-treated mice of all groups had significantly increased levels of human IL-15 (Figure 6a) and human granulocyte–macrophage colony-stimulating factor (hGM-CSF) (Figure 6b) compared with PLA-treated mice ($P = 0.027$ and 0.009, respectively, PLX-PAD vs PLA).

DISCUSSION

Despite the tremendous efforts invested in elucidating the mechanisms involved in the development of PE, as well as in identifying early biomarkers that may identify women at risk of developing PE, no therapeutics have yet been developed to treat it. The reluctance to testing novel therapies in pregnant women is understandable; however, with no new therapies the incidence of PE may continue to rise and negatively affect the lives of mothers and babies. In the present study we tested whether a cell therapy could ameliorate PE in mice.

Mesenchymal stem cells derived from adipose tissue, bone marrow, umbilical cords and placentas have cytoprotective and immunoregulatory properties, as well as low immunogenicity. Cell therapy with these cells has been reported to reduce the severity of a number of inflammation-related diseases in experimental animals. With respect to PE, Liu et al. [22] reported that mesenchymal stem cells isolated from the decidua of human placentas could reduce the PE-like features in mice given proinflammatory T-cells. The authors suggested that a reduction in TNFα probably contributed to the decrease in PE-like features in these mice. Although the data from this study and others are promising, a limiting factor is the potential of mesenchymal stem cells to differentiate. It is unclear whether these cells migrate to sites of injury during PE, including the vasculature, kidney and placenta, and become functioning cells, or whether they can cross the placental barrier.

PLX-PAD cells are mesenchymal-like, adherent stromal cells that have cytoprotective and immunoregulatory properties with very low immunogenicity, similar to mesenchymal stem cells; however, they do not have the same differentiation potential as mesenchymal stem cells. A previous study using PLX-PAD cells to treat hindlimb ischaemia in mice demonstrated that PLX-PAD cells were retained at the site of the

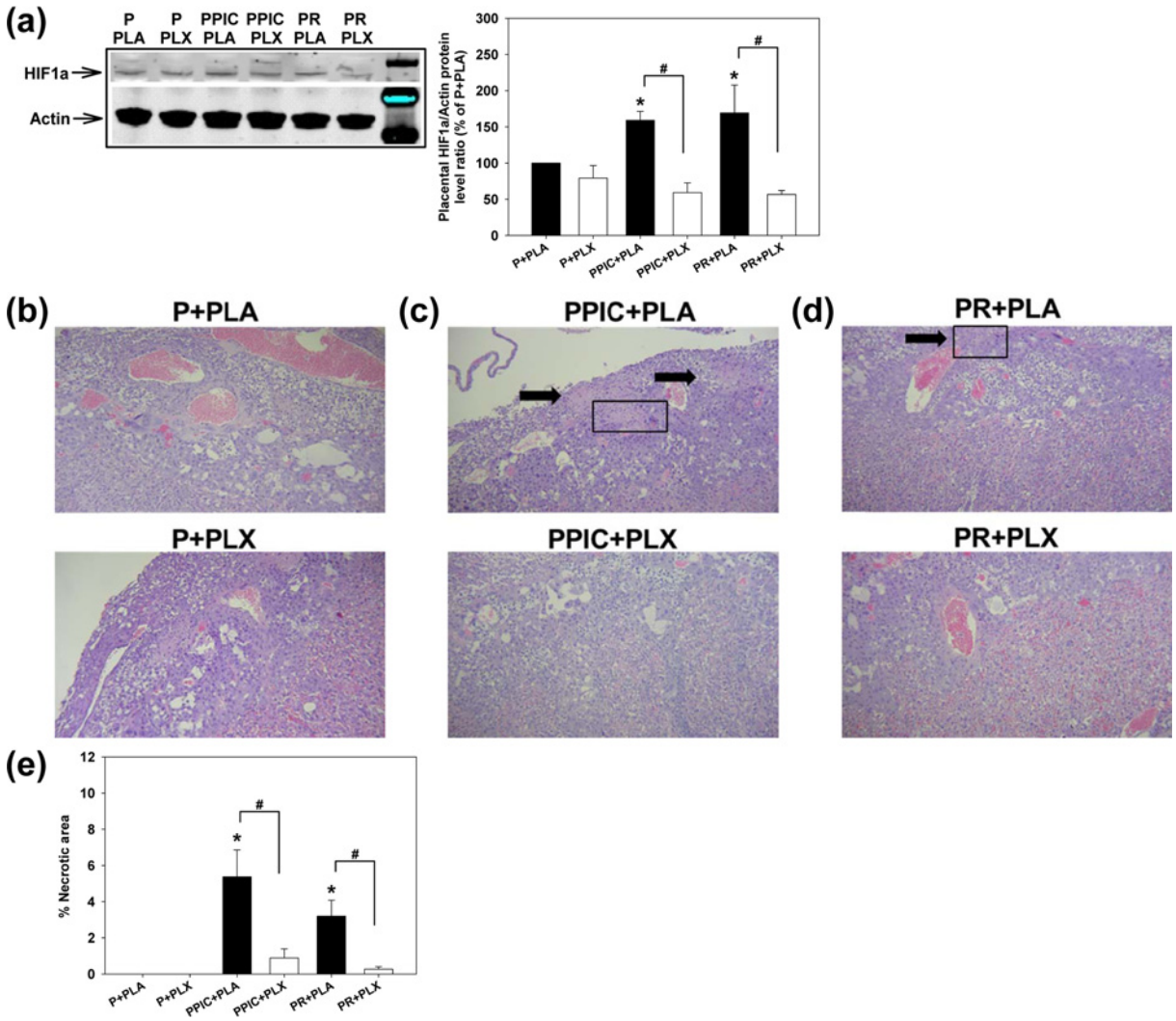

Figure 4 **PLX-PAD cells reduced placental injury in PE mice**

(**a**) HIF1α protein levels were increased significantly in placentas from PPIC and PR mice given the vehicle PLA; however, this was prevented by PLX-PAD cells (PLX) given on day 14 of pregnancy. $*P < 0.05$ vs P, $\#P < 0.05$ vs PLA of the same group. (**b**) Representative images of placentas stained with H&E from pregnant (P) mice treated with PLA (top panel) or PLX-PAD cells (bottom panel). (**c**) Representative images of placentas stained with H&E from pregnant mice treated with PPIC together with PLA (top panel) or PLX-PAD cells (bottom panel). Black arrowheads and the black box denote areas of necrosis. (**d**) Representative images of placentas stained with H&E from pregnant mice treated with PR along with PLA (top panel) or PLX-PAD cells (bottom panel). The black arrowhead and black box denote areas of necrosis. (**e**) Quantification of placental necrosis was performed using Image J and the total area of the 10× image was measured, followed by the necrotic area; the data area expressed as percentage necrotic area. $*P < 0.05$ vs P, $\#P < 0.05$ vs PLA of the same group. Results are expressed as means+S.E.M.s; $n = 6$–8 mice in each group. Images in (b) to (d) are 10× magnification; black box areas are shown at 40× magnification in Supplementary Figure S1.

intramuscular leg injection and remained within the body for 3 weeks before becoming undetectable [19]. The authors visualized the luciferase-expressing PLX-PAD cells after injection, and provided images showing that the cells remained in the leg muscle injection site and did not distribute to other organs in both Balb/c and non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice. Additional data generated by good laboratory practice, and used to support the testing of PLX-PAD cells in phase 1 studies for other diseases, used quantitative PCR and found that PLX-PAD cells did not migrate after intramuscular injection (data not shown). Therefore, it is highly unlikely that PLX-PAD cells migrate to the blood vessels, kidneys and placenta but instead secrete paracrine factors in response to the proinflammatory, pro-oxidant and ischaemic environment in which they are placed. Similar beneficial effects were seen when PLX-PAD cells were injected into the heart of mice after myocardial ischaemia [20]. The authors suggested that the cytoprotective, anti-inflammatory and proangiogenic factors secreted by the PLX-PAD cells could reduce myocardial infarct size and improve cardiac contractile function. In the present study, as well, the PLX-PAD cells were no longer detectable in the mice after 4 weeks, thus having presumably been eliminated. These

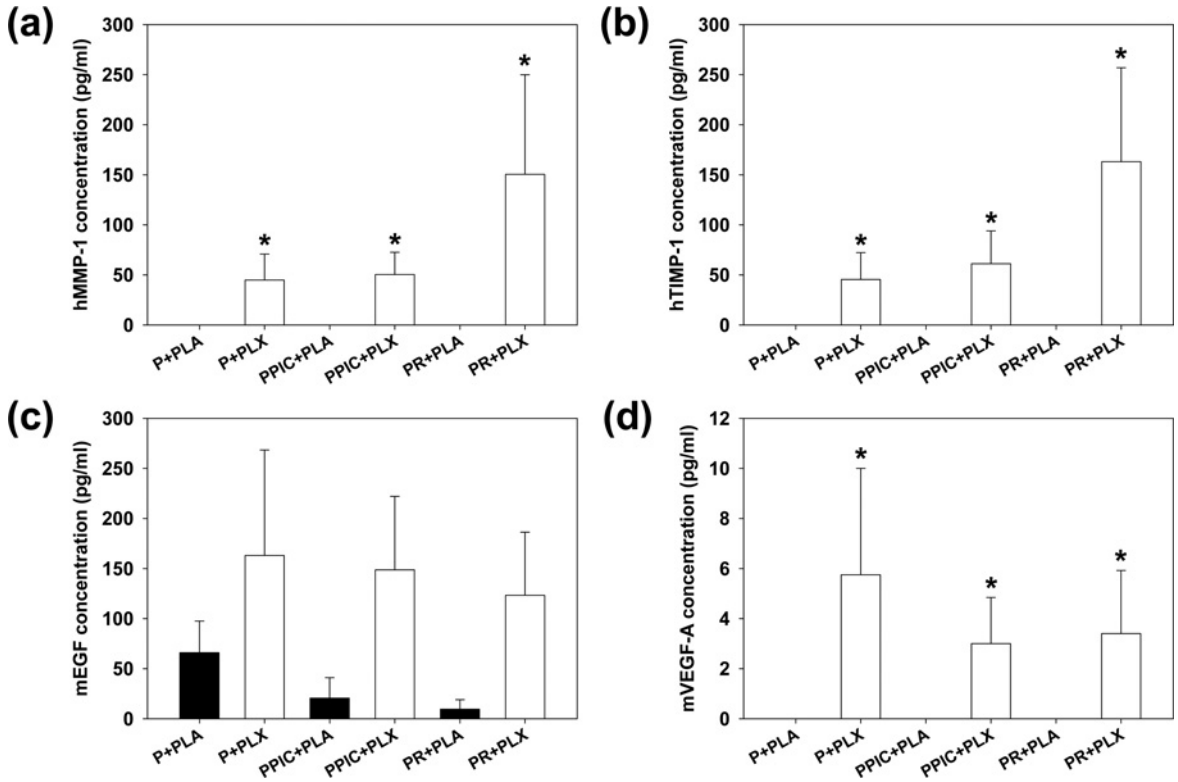

Figure 5 **PLX-PAD cells affect day 15 plasma cytokine profiles in mice**
Pregnant mice treated with P, PPIC or PR on days 13, 15 and 17 together with 1×10^6 PLX-PAD cells on day 14 had increased plasma levels of (**a**) human MMP-1, (**b**) hTIMP-1, (**c**) mEGF and (**d**) mVEGF-A, compared with PLA-treated mice. $*P < 0.05$ PLX-PAD cells vs PLA. Results are expressed as means+S.E.M.s; $n = 4$–5 mice in each group.

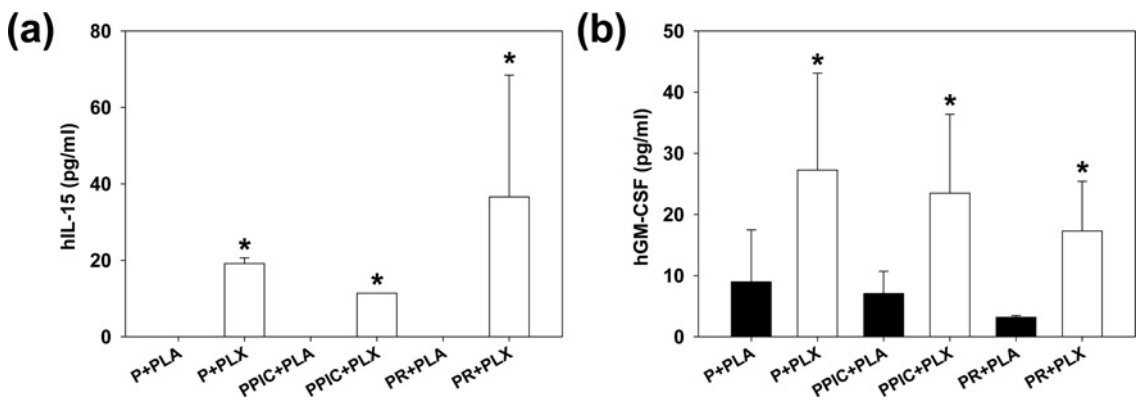

Figure 6 **PLX-PAD cells affect day 18 plasma cytokine profiles in mice**
Pregnant mice treated with P, PPIC or PR on days 13, 15 and 17 together with 1×10^6 PLX-PAD cells on day 14 had increased plasma levels of (**a**) human IL-15 and (**b**) hGM-CSF compared with PLA-treated mice. $*P < 0.05$ PLX-PAD cells vs PLA. Results are expressed as means+S.E.M.s; $n = 4$–5 mice in each group.

properties make PLX-PAD cells a viable candidate for the treatment of PE.

In our two models of innate immunity-induced PE, intramuscular injections of PLX-PAD cells administered on a single day could reduce all the measured PE-like features in these mice. In contrast, human adipose or bone marrow cells could not reduce the inflammation, hypertension or proteinuria in PPIC-treated pre-eclamptic mice. The reasons for this lack of effect of these stromal cells are unknown, but perhaps there was insufficient time for these cells to exert beneficial effects because they were injected on gestational day 14, the number of cells we injected was inadequate to overcome the TLR-induced immunity and inflammation or mesenchymal stem cells made up a small portion of these cells. Nevertheless, several important findings highlight the clinical potential of PLX-PAD cells. First, the gradual reduction in blood pressure, rather than a steep immediate drop,

should prevent any potential maternal hypotensive and low placental perfusion effects. Second, the blood pressure effects were dose dependent, suggesting that clinicians can tailor the therapy based on the blood pressure responses of the patient. Third, even though the PLX-PAD cells reversed the observed endothelial dysfunction, renal dysfunction and placental injury, presumably via the secretion of paracrine factors, there were no negative effects on the fetuses. Last, the large numbers of PLX-PAD cells that can be obtained from human placentas, their excellent safety profiles in clinical trials for other diseases and their ability to be administered as an allogeneic cell product without human leukocyte antigen (HLA) matching make them a promising therapeutic agent for the treatment of PE.

It is possible that PLX-PAD cells vary their release of factors based on the environment that they encounter. A subset of growth factors may be released early in a proinflammatory, pro-oxidant, ischaemic environment, whereas a different subset of factors may be released later. In the hindlimb ischaemia model, PLX-PAD cells were shown to have beneficial effects without the need for cell–cell contact, suggesting that it was the release of paracrine factors that led to the observed decrease in oxidative stress, endothelial inflammation and angiogenesis [19]. In the myocardial infarction model, VEGF and angiopoietin-1 were secreted by PLX-PAD cells under hypoxic culture conditions, whereas IL-10 and periostin were significantly increased by PLX-PAD cells *in vivo*, probably contributing to the observed improvement in myocardial structure and function [20]. Another report, utilizing mesenchymal stromal cells, demonstrated that they release factors that lead to activation of STAT3 and manganese superoxide dismutase in endothelial cells, which in turn limit oxidative injury [23]. Although it is possible that any or all of these factors contributed to the beneficial effects of PLX-PAD cell therapy in our PE models, we also observed significant increases in hMMP-1, hMMP-2 and hMMP-3, as well as hTIMP-1, on day 15 of gestation, 1 day after PLX-PAD cell administration. It is possible that these hMMPs, which degrade various forms of collagen, were released in response to the vascular, renal and placental injury in PE, thus limiting the fibrotic injury and improving organ function. Various studies have reported hMMP levels to be decreased, unchanged or increased in women with PE [24–28]. Although we cannot exclude some overlap between the detection of human and mouse proteins by ELISA, we did observe marked increases in mEGF and mVEGF-A in mice treated with PLX-PAD cells. It is well known that both these growth factors are decreased in PE, and the induction of these endogenous growth factors by human PLX-PAD cells may have also contributed to the beneficial effects of PLX-PAD cells [29–32]. By day 18 of gestation, once the hypertension and other pathologies had been diminished by PLX-PAD cell treatment, we observed that levels of human IL-15 and hGM-CSF were increased significantly in all mice given PLX-PAD cells. Both these cytokines provide signals for immune cell proliferation. Although some studies report increased levels of these cytokines in both the blood and placentas of women with PE [33–35], it is unknown whether their function at this time point is beneficial or detrimental. Decreased IL-15 has also been reported in placentas from women with PE, and IL-15 was demonstrated to induce $CD56^{bright}$ decidual natural killer (NK) cells to produce the anti-inflammatory cytokines IL-10 and IL-13 [36]. IL-15 also inhibits apoptosis and increases angiogenesis [37,38]. These proliferation signals may lead to polarization and expansion of immunoregulatory immune cells including regulatory T-cells, M_2 macrophages, myeloid-derived suppressor cells and uterine/decidual NK cells, in addition to other effects [39,40]. Therefore, our data suggest that the resolution of TLR-induced inflammation, by the release of anti-inflammatory cytokines and growth factors from PLX-PAD cells, prevented vascular, renal and placental injury and dysfunction, leading to decreased blood pressure and urinary protein excretion during pregnancy.

Limitations of the present study include the chosen mouse model, which represents the induction of PE-like features induced by innate immune system activation and inflammation during pregnancy, one likely cause of some forms of PE. It is possible that the differences between the mouse and human immune systems affect the results of this study. Additional studies are under way to examine whether PLX-PAD cells can also ameliorate the PE-like features induced by angiogenic imbalance, another likely cause of some forms of PE. In addition, PLX-PAD cells are being tested in clinical trials for other diseases and have shown a safe profile in more than 100 patients; however, there may be a small risk that the pregnancy and/or the pre-eclamptic state make women susceptible to negative effects, not observed thus far in non-pregnant women or pregnant animals during this study or independent reproductive toxicology studies.

In our innate immunity-mediated model of PE, we have demonstrated that treatment with PLX-PAD cells, even after the development of PE-like features, could reduce the observed hypertension, proteinuria, endothelial dysfunction, inflammation and placental injury. The beneficial effects of this cell therapy may be due to the immunoregulatory, anti-inflammatory and anti-necrotic factors released from the PLX-PAD cells. PLX-PAD cells may therefore be useful in the treatment of PE in women.

CLINICAL PERSPECTIVES

- Currently there is no treatment for PE, a hypertensive disorder of pregnancy.
- We tested whether PLX-PAD cells could reduce PE-like features in two mouse models of PE. A single treatment of PLX-PAD cells could decrease SBP, urinary protein excretion, endothelial dysfunction and placental injury in both models.
- These preclinical data demonstrate the safety and efficacy of PLX-PAD cells for the treatment of experimental PE. It is our hope that PLX-PAD cells, currently in clinical trials for other indications, will be entered into clinical trials for PE in the near future.

AUTHOR CONTRIBUTION

Piyali Chatterjee performed the PLX-PAD cell preparations, mouse injections, blood pressure measurements, imaging studies and fetal development analyses. Valorie Chiasson assisted with the studies and performed the immunoblotting, day 18 mouse plasma protein array, and urinary protein and creatinine assays. Lena Pinzur

contributed to experimental designs (PLX-PAD cell injection timing, dosing, control cells, plasma collection timing and cytokine panel design). Shani Raveh performed the Luminex assays of plasma on gestational day 15. Eytan Abraham and Liat Flaishon contributed to experimental designs (PLX-PAD cell injection timing, dosing and control cells). Kathleen Jones provided the blinded histological analyses. Kelsey Bounds assisted with the biochemical measurements. Racheli Ofir contributed to experimental designs (PLX-PAD cell injection timing, dosing, control cells, plasma collection timing and cytokine panel design) and oversaw the direction of the Pluristem site at later stages of the project. Ayelet Chajut contributed to experimental designs (PLX-PAD cell injection timing, dosing, control cells, plasma collection timing and cytokine panel design) and directed the Pluristem site at the early stages of the project. Brett Mitchell performed the blood pressure and vascular reactivity measurements and oversaw the direction of the project in Temple, TX, U.S.A. Piyali Chatterjee and Valorie Chiasson contributed equally to this paper.

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