Maternal microchimerism and cell-mediated immune-modulation enhance engraftment following semi-allogenic intrauterine transplantation

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Abstract

Successful intrauterine hematopoietic cell transplantation (IUT) for congenital hemoglobinopathies is hampered by maternal alloresponsiveness. We investigate these interactions in semi-allogenic murine IUT. E14 fetuses (B6 females × BALB/c males) were each treated with 5E+6 maternal (B6) or paternal (BALB/c) bone marrow cells and serially monitored for chimerism (>1% engraftment), trafficked maternal immune cells, and immune responsiveness to donor cells. A total of 41.0% of maternal IUT recipients (mIUT) were chimeras (mean donor chimerism 3.0 ± 1.3%) versus 75.0% of paternal IUT recipients (pIUT, 3.6 ± 1.1%). Chimeras showed higher maternal microchimerism of CD4, CD8, and CD19 than non-chimeras. These maternal cells showed minimal responsiveness to B6 or BALB/c stimulation. To interrogate tolerance, mIUT were injected postnatally with 5E+6 B6 cells/pup; pIUT received BALB/c cells. IUT-treated pups showed no changes in trafficked maternal or fetal immune cell levels compared to controls. Donor-specific IgM and IgG were expressed by 1%-3% of recipients. mIUT splenocytes showed greater proliferation of regulatory T cells (Treg) upon BALB/c stimulation, while B6 stimulation upregulated the pro-inflammatory cytokines more than BALB/c. pIUT splenocytes produced identical Treg and cytokine responses to BALB/c and B6 cells, with higher Treg activity and
1 | INTRODUCTION

Intrauterine hemopoietic stem cell transplantation (IUT) is applicable to several congenital genetic conditions, including the major hemoglobinopathies, hereditary immunodeficiencies, and inborn errors of metabolism.1-3 Preclinical fetal therapy research has demonstrated by proof of principle that intervening at an early developmental stage offers numerous benefits leveraging on fetal immune tolerance, stoichiometry, and availability of hemopoietic niches.4-6 Translation from experimental models to humans has been less successful, with transplantation in all conditions, apart from the immunodeficiencies, failing to achieve sustained therapeutic effect.7 An evolving strategy is to render the fetus tolerant with early exposure to donor cells and to further reinforce immunity and engraftment with postnatal transplantation of the same cells.5,8 This approach is employed in a clinical trial of intrauterine transplantation to treat osteogenesis imperfecta.9 While allogenic fetal mesenchymal stem cells are hypoimmunogenic, hemopoietic stem and progenitor cells (HSPC) are immunogenic, requiring myeloablation and immunosuppression for postnatal transplantation of the same cells.5,8 This approach is employed in a clinical trial of intrauterine transplantation to treat osteogenesis imperfecta.9 While allogenic fetal mesenchymal stem cells are hypoimmunogenic, hemopoietic stem and progenitor cells (HSPC) are immunogenic, requiring myeloablation and immunosuppression for postnatal transplantation.10,11 Recent works have better delineated the mainly immune engraftment barriers encountered in IUT, of which sensitized trafficked maternal cells are a significant component, provoking graft rejection via the adaptive immune system.12-15 This may be circumvented by transplanting semi-allogenic maternal bone marrow (BM)-derived HSC which demonstrate better tolerance and more efficient engraftment,6,9 a strategy used in a current clinical trial for alpha thalassemia major (NCT02988698) which has successfully reported the delivery of the first two treated infants. However, we were unable to replicate the same engraftment in the non-human primate (NHP) model and thus questioned the influence of this maternal microchimerism (MMc) on donor cell survival and engraftment, as well as the influence of semi-allogenic donor cells from either the paternal or maternal donor on the responsiveness of trafficked maternal cells.16 We investigated if maternal donor cells were better tolerated and engrafted more efficiently than paternal cells due to the anticipated lower immune-responsiveness of trafficked maternal immune cells resulting in a more favorable influence on engrafted donor cells.12,14 We used a cross-bred murine model (CD57BL/6 x BALB/c) for a direct comparison of maternal (mIUT) and paternal donor cell IUT (pIUT), interrogating engraftment, maternal immune microchimerism, and fetal immune response (Figure 1A).

2 | MATERIALS AND METHODS

2.1 | Reagents and antibodies

Roswell Park Memorial Institute Medium (RPMI 1640), fetal bovine serum (FBS), non-essential amino acids, GlutaMAX, sodium pyruvate, penicillin-streptomycin antibiotics, and ionomycin were obtained from Gibco (Thermo fisher Scientific). Diprotin A was obtained from Sigma-Aldrich (St. Louis, MO, USA). Mitomycin C was obtained from ALPS Pte Ltd (National University Hospital Pharmacy, Singapore). Percoll and Ficoll-Paque PLUS were obtained from GE Healthcare, USA. FastStart Universal SYBR green master mix (Roche Life Science, Germany) was obtained from Sigma-Aldrich. DNA oligos for qPCR were obtained from Integrated DNA Technologies (IDT, Singapore). RBC lysis buffer was obtained from Biologend (San Diego, CA, USA). Cytofix/Cytoperm solution, Perm/Wash buffer, staining buffer, brilliant stain buffer, and fixable viability stain 620 were obtained from BD Biosciences (San Jose, CA, USA). The list of flow cytometry antibodies used can be found in Supplementary Table S1.

2.2 | Animal experiments

Inbred strains CD45.2 BALB/c (H-2Kb) and CD45.2 C57BL/6 (H-2Kb, referred to as B6) were obtained from In Vivos (Singapore), and CD45.1 C57BL/6 strain mice were purchased.
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from The Jackson Laboratory (Maine, USA) and maintained in a specific pathogen-free facility at NUS. BALB/c males and B6 females were time-mated for IUT at E14. To investigate whether maternal cell trafficking and engraftment was strain-specific, BALB/c females and B6 males were time-mated separately for E14 IUT. All mouse experiments were performed according to IACUC (Institutional Animal Care and Use Committee) approved protocols (BR16-1203 and R16-1200) at the National University of Singapore (NUS), Singapore.

2.3 | In utero hematopoietic cell transplantation (IUT)

Adult B6 or BALB/c mice aged ~ 1.2-1.5 years old were sacrificed and their femurs, tibias, and iliac leg bones flushed for BM to harvest mononuclear cells (BM-MNC) were prepared as previously described. In our initial experiments, donor BM-MNC were treated with CD26 inhibition. Maternal donor cell IUT (mIUT) was performed using B6 BM-MNC (CD45.1, H-2Kb) and BALB/c BM-MNC was administered in paternal IUT (pIUT). IUT was performed under isoflurane anesthesia on E14 via midline laparotomy. Uterine horns were exteriorized, and each fetus was injected with 5E+6 BM-MNC cells in 10 μL of phosphate-buffered saline (PBS) intrahepatically (IH) using a 100 μL of Hamilton Microliter syringe and needle (GR, Switzerland) under a stereomicroscope (Olympus, Singapore). The abdomen was then stitched back with absorbable 6.0 polyglactin 910 (Vicryl, Ethicon) after replacing the uterine horns as previously described. After littering, pups were nursed by their mothers and weaned.
at 4 weeks. Fetal cells were harvested from F1 cross-bred pups from E12 to 24 weeks. Donor cell chimerism (DCC), trafficked maternal cell microchimerism (MMc), and fetal immunological responses were assessed at predetermined time points from MNC isolated from the harvested peripheral blood (PB), bone marrow (BM), liver, spleen, and lymph nodes (LN) of IUT recipients. Controls were age-matched naïve pups which were not transplanted in utero. A separate cohort of IUT-treated hybrid pups were postnatally boosted with 5E+6 cells/50 μL BM-MNC (4 weeks after birth) by tail vein injection while control pups received saline.

2.4 | Isolation of MNC and FACS analysis

PB and BM-MNC were prepared by density gradient centrifugation using Ficoll-Paque PLUS density gradient media (GE Healthcare, NJ, USA) as described previously.19 Liver MNC were prepared as described17: digestion by collagenase IV (0.04%, 10 minutes at 37°C), passing through 70 μm nylon mesh, centrifugation (150g × 5 minutes) to remove debris, pelleting of cells (400g × 5 minutes), resuspension of cell pellet in 40% of Percoll solution (overlaid on 70% Percoll), gradient centrifugation (800g × 25 minutes), and harvesting MNC from the Percoll interface. Spleen was minced and mashed through a 70 μm cell strainer producing a single-cell suspension. The cell pellet was resuspended in 40% Percoll, overlaid on 70% Percoll, gradient centrifugation (800g × 25 minutes), and pelleting of cells (400g × 5 minutes), then resuspended in 40% Percoll, overlaid on 70% Percoll, centrifugation (800g × 5 minutes), pelleting of cells (400g × 5 minutes), and resuspension of cell pellet in 40% of Percoll solution (overlaid on 70% Percoll), gradient centrifugation (800g × 25 minutes), and harvesting MNC from the Percoll interface. Spleen was minced and mashed through a 70 μm cell strainer producing a single-cell suspension flow-through which was centrifuged (400g × 5 minutes), the cell pellet was subjected to RBC lysis and washed with PBS. LN were excised from the axillary, brachial, and inguinal regions, minced and digested with collagenase IV (0.04% × 20 minutes at 37°C), passed through a 70 μm cell strainer, centrifuged (400g × 5 minutes), and FACS sorted (FACS). Approximately 1E+6 cells/tube were initially stained with fixable viability stain 620, followed by FC blocking and a cocktail of FACS antibodies diluted in brilliant stain buffer (BD Biosciences, USA). All stained cells were washed and analyzed using BD LSRFortessa flow cytometer (BD Biosciences, USA) from NUS School of Medicine core facilities. All raw data files were compensated and analyzed using FlowJo software (FlowJo LLC, OR, USA). Immune profiles for T cells (CD3, CD4, CD8, NK1.1), B cells (CD19), and APC (CD11c) were calculated, after gating to segregate donor cells, fetal cells, and maternal trafficking cells (Supplementary Figure S1A). Fetal cells/tissues represent cells analyzed from fetal organs of cross-bred F1 pups/offspring/recipients harvested from E-12 to 24 weeks with or without IUT.

2.5 | Detection of donor-specific antibodies

PB from IUT-treated pups was obtained by retro-orbital puncture12 and serum isolated by high-speed centrifugation and stored at −80°C until used for experiments. In postnatally boosted mice, PB was collected before and 2 weeks after boosting with BM-MNC. Sensitized sera were produced by immunizing B6 adults with IP injections of 2E+7 BALB/c splenocytes and LN MNC at days 0 and 7. Serum was collected on day 14 and other time points. BALB/c splenocytes were used for pIUT serum samples and B6 splenocytes were used for mIUT serum samples. 5E+5 splenocytes were pre-incubated with anti-CD16/32b Fc bloc, incubated for 45 minutes with serum at dilution ratios of 1:200, 1:20, 1:2, and 1:1,12 washed twice to remove the excess serum and incubated for 45 minutes with secondary anti-mouse IgG and IgM. The stained cells were analyzed using flow cytometry to determine the MFI of IgM and IgG staining on CD19-negative cells. The relative MFI was normalized to that of lymphocytes that were not exposed to serum and expressed as fold changes. Naïve serum was collected from age-matched cross-bred pups of uninterrupted pregnancies.

2.6 | Assessment of T cell activity

T cell activity was evaluated by performing mixed lymphocyte reaction (MLR) assays. Responder splenocytes from IUT-treated and naive pups (from uninterrupted pregnancies) were isolated in PBS containing 2 mM of ethylenediaminetetraacetic acid (EDTA), RBC-lysed, and washed with PBS. The cell pellet was resuspended in 40% Percoll, overlaid on top of 70% Percoll solution and then, centrifuged at 800g for 25 minutes without brake. MNC harvested from the Percoll interface layer were washed twice with RPMI 1640 containing 10% of fetal bovine serum (FBS), counted, and seeded to 96-well U-bottom plates at 1.5E+5 cells/well) in 100 μL of RPMI1640 supplemented with 2 mM of glutamine, 1% (v/v) of nonessential amino acids, 1% (v/v) of sodium pyruvate, penicillin (50 U/mL), and streptomycin (50 μg/mL), and 10% (v/v) of heat-inactivated serum (Gibco, Life Technologies, Carlsbad, CA, USA). Splenocytes and LN MNC were isolated from either BALB/c or B6 and used together as stimulator cells, treated with mitomycin C (50µg/ml) and added to responder splenocytes (3E+5 cells/well).20 Cultures were harvested after 72 hours of incubation at 37°C, and cells were analyzed by FACS for T cell subset surface markers (CD4, CD8, CD25, CD62L, and CD44) and intracellular marker FOXP3 using BD Cytofix and Cytoperm. Cells were harvested for RNA extraction used for cytokine gene expression by qPCR.
2.7 | Cytokine expression by qPCR

Total RNA was extracted using RNeasy kit (Qiagen, cat. #74104) with the inclusion of on-column DNase I digestion (Qiagen, cat. #79254) to remove genomic DNA contamination. RNA was quantified by Nanodrop 1000 spectrophotometer (Thermo controller Scientific, NJ, USA). Reverse transcription qPCR was performed as previously described with Applied Biosystems 7500 Fast Real-Time PCR system (Thermo controller Scientific, NJ, USA); in brief cDNA was synthesized using Superscript III (Thermo controller Scientific, NJ, USA), diluted in nuclease-free water prior to qPCR amplification, and ~20 ng per sample used with FastStart universal SYBR green master mix (Roche Life Science, Penzberg, Germany) for qPCR of 40 cycles (denaturation at 95°C for 10 seconds, annealing, and extension at 59°C for 30 seconds). Melting curve analysis was performed after amplification to exclude nonspecific amplification or primer-dimer formation. No template control was included in qPCR analysis and all gene expression values were normalized with endogenous control GAPDH. Relative quantification of each target gene studied was calculated by the ∆∆CT method. The list of qPCR primers used for cytokine gene expression can be found in Supplementary Table S2.

2.8 | Statistical analysis

Continuous data were analyzed by Analysis of variance (two-way ANOVA) with Tukey’s multiple comparisons test with a single pooled variance and multiple t tests for the comparisons of individual parameters. Statistical significance was determined at α = 5.0%. Values are expressed as mean ± SD. Pearson correlation coefficient was also used assuming linear relationships between the variables tested. Analyses were performed with GraphPad Prism version 9 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.

3 | RESULTS

3.1 | Paternal IUT resulted in higher DCC than maternal IUT with multilineage engraftment of hemopoietic donor cells

We performed intrahepatic IUT at E14 in cross-bred fetuses (B6/CD45.2/H2kb+females [B6] × BALB/c /CD45.2/H2kd+males [BALB/c] Figure 1A), a model that allowed us to independently track maternal (CD45.2/H2kb+/d-), fetal (CD45.2/H2kb+/d+), and donor cells (Figure 1B). Fetuses received donor cells either from maternal B6 (coisogenic CD45.1/H2kb+/d-) or paternal BALB/c (CD45.2/H2kb-/d+) bone marrow mononuclear cells (MNC); all cell groups were identified on flow cytometry by expression of CD45.1/45.2 and/or H2k antigens. Chimerism in F1 progeny was defined as donor cell engraftment >1% of total recipient MNC, and chimerism frequency indicated the percentage of injected pups which were chimeras (>1% engraftment). CD26 inhibition of donor cells with Diprotin A, demonstrated to increase homing and engraftment of allogenic IUT, resulted in substantial cell loss, with no differences in chimerism with (n = 26) or without inhibition (n = 16, Figure 1C). Higher MMc (trafficked maternal cells; maternal microchimerism) and fetal CD3, CD19, and CD8 followed IUT with CD26 inhibition; subsequent IUT were performed without CD26 inhibition. Perinatal survival was ~68% among all mIUT (n = 83/122) and ~76% among all pIUT litters (n = 46/80), with ~28% pregnancy resorption or postnatal cannibalization in both groups, while 100% of saline-injected litters survived. Donor cell chimerism (DCC) in pups transplanted without CD26 inhibition was assessed monthly from 4 postnatal weeks (pw, Figure 1D). A total of 41.0% of these mIUT recipients were chimeras (n = 16/39); DCC was 0.2 ± 0.1% overall (all animals) and 3.0 ± 1.3% among chimeras (>1% donor cells) from 4 to 24 pw, with the highest levels observed at 8 pw (3.8 ± 4.5%, Figure 1D). Most animals demonstrated liver chimerism; fewer recipients had detectable chimerism in PB or BM. A total of 75.0% of pIUT recipients (without CD26 inhibition) were chimeras (n = 24/32), with a mean overall DCC of 2.4 ± 1.3% in all animals (Figure 1D), significantly higher than mIUT chimerism, and 3.6 ± 1.1% among chimeras only, with highest levels detected at 16 pw (5.2 ± 5.0%). Overall DCC levels in PB and BM were higher in pIUT recipients than observed in mIUT; in PB this was significant at all time points except 12 pw and highest at 4 pw (4.4 ± 1.9%, Figure 1E). BM chimerism in these pIUT recipients was 3.7 ± 2.0% over all time points, with a peak of 5.9 ± 5.5% at 16 pw; in contrast, BM chimerism of all mIUT recipients was 0.06 ± 0.03% at all time points (Figure 1E). We next investigated if transplanted donor cells retained their multilineage hemopoietic and immune profile following engraftment. Pretransplant composition of maternal B6 and paternal BALB/c donor cells showed similar populations of short- and long-term repopulating hemopoietic stem and progenitor cells (HSPC). BM-MNC were characterized for lineage and hematopoietic stem cell markers by flow cytometry. Freshly isolated BM-MNC were stained for lineage markers TER119 (erythroid lineage), CD11b (myeloid lineage), GR1 (granulocytes) to gate out lineage-positive cells (TER119+, CD11b+, and GR1+), and HSC markers Sca-1,
c-Kit, CD48 (short-term HSC), and CD150 (long-term HSC) to characterize the remaining lineage-negative population (Lin-). After excluding cells with lineage markers, approximately 40%-60% of donor BM-MNC remained (Lin- cells), and of these, 0.2% were Sca1+cKit+ (LSK cells), 0.1%-0.3% were LSK CD48+/CD150- (short-term HSPC), and 0.03%-0.09% were LSK CD48-/CD150+ (long-term HSPC). B6 cells had higher Sca1+population than BALB/c (30.5% vs 1.0%, \( P < .001 \)). Among Sca1- cKit- non-LSK cells, CD48+and CD150+cells comprised 45%-62% and 0.1%-0.2%, respectively, and 24%-30% were CD48+CD150+ double positive (Figure 2A). BALB/c cells showed higher CD48+cells in the Sca1- cKit- population (62.2% vs 45.0%, \( P < .001 \)). Donor cells engrafted in fetal hematopoietic organs (PB, BM, spleen, and liver) retained their multilineage hemopoietic profile, expressing cKit, Sca1, CD48, CD150, and erythroid-specific marker TER119 (n = 10). The typical HSPC profile of transplanted cells was similar in both mIUT

**FIGURE 2** Characterization of B6 bone marrow MNC donor cells. A, Lineage negative (Lin-) population (CD11b-, GR1-, and TER119- cells) were analyzed for HSC markers Sca1 and c-Kit. Sca1+c-Kit+Lin- double positive cells were gated for short-term HSC (CD48) and long-term HSC markers (CD150). Most of these cells were CD48+ and CD150+ double positive cells from the Sca1- c-Kit- double negative population. B, Engrafted donor cells demonstrated a multilineage immune profile after harvesting from all hemopoietic organs at 4-24 pw. C, Compared with the current breeding pair, no significant differences in MMc were observed in progeny of BALB/c females crossed with B6 males.
(LSK 0.2 ± 0.4%, LSK CD48+0.02 ± 0.05%, Sca1- cKit-CD48+56.1 ± 9.3%, Sca1- cKit- CD150+0.5 ± 0.8%, and TER119 6.6 ± 7.6%) and pIUT recipients (LSK 1.4 ± 1.8%, LSK CD48+0.9 ± 1.1%, Sca1- cKit-CD48+31.6 ± 24.7%, Sca1- cKit- CD150+5.0 ± 7.6%, and TER119 1.0 ± 1.4%). Engrafted donor cells also demonstrated a multilineage immune profile, with T cells, B cells, NK, and antigen-presenting cells harvested from all hemopoietic organs at all time points from 4 to 24 pw (Figure 2B). There were no differences in hemopoietic and immune profiles between male and female pups. No significant differences in MMc were observed in progeny of BALB/c females crossed with B6 males (E16 to Day 0 only, Figure 2C).

3.2 | Naïve (uninjected) pups displayed long-term MMc and responded vigorously on postnatal exposure to paternal splenocytes

We examined the immune profiles of naïve (uninjected) cross-bred F1 progeny (B6 females x BALB/c males) by tracking CD45.2/H2kb+/d- (maternal) and CD45.2/H2kb+/d+ (fetal) cells; these served as comparisons for IUT-treated pups. MMc was detectable from E12 until 24 pw in all hematopoietic organs, heart, and lungs, ranging from 0.1% to 0.8% of total MNC in the naïve fetus (n = 64). Maternal CD4, CD8, and CD19 cells were first detected in fetal liver at E12 (Figure 3A); these together with NK1.1 and CD11c+cells...
were detected in liver, BM, spleen, and thymus by E18. By 1 pw CD19 was the most frequent maternal cell found in all organs (3.7% in lymph node: LN, 25.6% in bone marrow: BM), declining in levels until 24 pw. CD4 and CD8 levels peaked between E18 and birth and were last detected in PB (2.2%-4.4%) at 4-8 pw. Peak NK1.1 levels were observed at E18 and persisted in fetal tissues until 24 pw. CD11c formed <1% of total maternal MNC, observed in all organs from birth until 4 pw (0.05%-0.2% in solid organs) and no longer detected by 8 pw. Attempts to isolate fetal immune cells at E12 were unsuccessful. Fetal CD19 and NK1.1 cells were detected in naïve progeny from at least E18; CD19 persisted at 20%-80% of total fetal MNC from 1 to 24 pw while NK1.1 cells dropped to 0.02%-0.15% from 4 pw (Figure 3B). Stable plateau was reached in fetal CD4 (17.2%-43.1%) and CD8 (1.1%-14.3%) by 4 pw which persisted until 24 pw. CD11c cells were observed from 1 pw onwards.

We analyzed the functional responses of naïve splenocytes harvested from untreated F1 progeny when exposed postnatally to maternal and paternal donor cells. Naïve splenocytes were harvested at 4 pw and stimulated with mitotically inactivated splenocytes from pure-bred B6 and BALB/c donors in a MLR assay, with unstimulated naïve splenocytes as controls. Proliferation of reactive T cells and associated cytokine expression were analyzed by FACS and qPCR. Stimulation with B6 splenocytes (n = 6) caused proliferation of naïve CD8 effector, regulatory, and memory T cells and CD4 regulatory T cells (Treg) and CD25+Treg, but caused no changes to CD4 memory and effector subsets compared to controls (Figure 3C). Stimulation with BALB/c splenocytes (n = 6) upregulated naïve CD4 and CD8 Treg and CD25+Treg, in addition to CD4 and CD8 stem cell-like memory (T-SCM), central memory (T-CM) and effector memory (T-EM) T cells, and CD4 and CD8 effector T cells (Teff, Figure 3C). B6 stimulation produced an overall down-regulation of pro-inflammatory and certain immunosuppressive cytokines compared with unstimulated controls, though there was upregulation of pro-inflammatory CSF2 and IFNγ and immunosuppressive IL10 (Figure 3D). BALB/c stimulation upregulated immunosuppressive FOXP3 and most pro-inflammatory/pro-immune cytokines, particularly those associated with Th17 helper cells (IL3, IL12a, IL22, CSF2, and IFNγ), more than B6 stimulation (Figure 3E). Most immunosuppressive cytokines (IL10, TGFβ, IL16, and IL27) were downregulated compared to unstimulated controls.

3.3 | Chimeras demonstrated a different pattern of MMc and fetal immune profile than non-chimeras

We interrogated MMc and fetal immune cell profiles of all pIUT and mIUT recipients in PB and BM. MMc (CD45.2/H2kb+/d-) was present at stable levels in all organs of IUT-treated pups (mIUT: 0.4%-1.3%, n = 83; pIUT: 0.06%-3.7%, n = 46, Figure 4A). The immune profiles of MMc in naïve, mIUT, and pIUT offspring were similar (Figure 4B). In the cohort receiving donor cells without CD26 inhibition, pIUT offspring (n = 32) demonstrated higher levels of trafficked maternal CD3, CD8, and NK1.1 cells, and lower CD19, in PB and BM than mIUT recipients (n = 39) (Figure 4C,D). Levels of fetal CD3, CD4, CD8, and NK1.1 cells were higher, and CD19 levels lower in PB of pIUT compared to mIUT pups (Figure 4E). In BM, pIUT pups had lower fetal CD3 and higher CD19 (Figure 4F). Among chimeras alone, we observed apparent loss of MMc over time in mIUT offspring (n = 16), although this was not different from pIUT chimeras (n = 24); mIUT chimeras also had higher CD19 at 8 pw (58.23% vs 8.68%, P < .0001, Figure 5A), and higher fetal CD19 at 8 pw (63.21% vs 36.97%, P < 0.02) than pIUT (Figure 5B). Compared to non-chimeras, chimeric pIUT pups had higher levels of trafficked maternal CD3, CD8 cells and lower CD19 cells in PB, BM (indicated by ** in Figure 5C; higher CD19 levels in BM at 12 pw), liver and LN (Supplementary Figure S1B). There were higher recipient CD3, CD4, and CD19 levels in offspring PB, BM (indicated by ** in Figure 5D; lower CD19 in BM at 8 pw) and liver. mIUT chimeras had higher maternal CD19 (24.77% vs 1.22% at 12 pw, Figure 5E), and higher fetal CD3 (36.80% vs 17.89%, 8 pw) and CD19 (75.73% vs 44.90%, 12 pw) than non-chimeras (Figure 5F).

3.4 | Microchimerism of maternal and fetal CD11c correlated strongly with donor cell engraftment in hemopoietic organs of IUT offspring

Among mIUT recipients there was only moderate or weak correlation (Pearson’s r < 0.8) between overall DCC and MMc in female offspring, while males showed strong correlation (Pearson’s r ≥ 0.8) between DCC and MMc in PB and BM (P < .001). Both female and male pIUT recipients demonstrated strong correlation (Pearson’s r ≥ 0.8) between PB and BM engraftment and MMc in BM. Although statistical significance was not always reached, mIUT showed negative correlation between PB and BM engraftment and maternal CD8 (r = -0.8, P = .2) and CD19 (r = -0.8, P = .6), with positive correlation between BM engraftment and maternal NK1.1 and CD11c (r = 1.0, P < .0001). In mIUT and pIUT recipients, higher PB and BM engraftment correlated with higher fetal CD19, CD3, and CD11c (Pearson r 0.8-0.9, P < .03) in mIUT recipients showed positive correlation between MMc and B6-specific fetal CD4 CD25+Tregs found in fetal LN
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(B) Maternal microchimerism (MMc) in miUT (n = 83) and pIUT recipients (n = 46) in all organs. A, MMc distributed in all organs of miUT and pIUT recipients, with trends toward higher levels in pIUT recipients. Values expressed as box and whiskers, min to max. B, Multilineage profile of MMc observed in naïve (untreated), miUT and pIUT offspring in PB, BM, and LN. No differences were observed in MMc levels across these groups. Two-way ANOVA, $\alpha = 0.05$, values expressed as mean. PB peripheral blood, BM bone marrow, and LN lymph node. C, Percentage of MMc in PB and (D) BM of pIUT offspring (open symbols, n = 32) show higher levels of maternal CD3, CD8, and NK1.1 cells and lower CD19 cells compared to miUT offspring (filled symbols, n = 39). The fetal immune profile in PB shows higher levels of fetal CD3, CD4, CD8, and NK1.1 cells and lower CD19 levels in pIUT compared to miUT recipients (E), and in (F) BM shows lower fetal CD3 and higher CD19 in pIUT recipients.

**FIGURE 4**  

(Pearson’s r 0.81, $P = .2$) and negative correlation in fetal spleen (Pearson’s r $-0.82$, $P = .2$), while pIUT recipients showed no or weak correlation between MMc and BALB/c-specific fetal CD4 CD25+Tregs in all organs (Pearson’s r $-0.6$ to 0.4).

3.5 | Maternal and paternal IUT recipients did not express donor-specific antibodies

To interrogate tolerance to donor cells, we first boosted a new cohort of cross-bred IUT recipients with the same donor
cells at 4 pw. Each offspring received $5 \times 10^6$ BM-MNC of either donor B6 cells ($n = 18$), BALB/c cells ($n = 12$) or saline ($mIUT, n = 12, pIUT, n = 11$). We did not perform pre-transplantation busulfan conditioning despite its facilitation of higher engraftment via hemopoietic niche clearance because of the expected immune microenvironment alterations, detrimental to our study of immune cell responsiveness. Because of this, there was no change in chimerism after postnatal boost during the observation period. There were no differences in maternal immune microchimerism in all organs between cell-boosted and saline-injected mice in either mIUT ($n = 16$) or pIUT ($n = 24$) recipient PB and BM combined. Individual values are represented on the graphs. A, mIUT chimeras had higher trafficked maternal CD19 at 8 pw (mean 58.23% vs 8.68%, $P < .0001$, Figure 3E), and (B) higher fetal CD19 at 8 pw (mean 63.21% vs 33.62%, $P < .0001$) and 12 pw (mean 75.73% vs 36.97%, $P = .02$) than observed in pIUT recipients. C, pIUT chimeras (denoted by C at the base of the bar graphs) showed higher percentage of maternal CD3 and CD8 cells, and lower CD19 cells, compared to non-chimeras (denoted by N at the base of the bar graphs) in PB and BM, and (D) higher percentage of fetal CD3, CD4, and CD19 in PB and BM than non-chimeras.

E, Maternal CD19 percentage levels were higher in mIUT chimeras (all organs combined) compared to non-chimeras, as were (F) fetal CD3 and CD19 levels. Multiple t test, $\alpha = 0.05$, values expressed as mean ± SD. PB: peripheral blood, BM: bone marrow.

**FIGURE 5** Comparison of trafficked maternal and fetal immune cell responses following IUT. A, MMc and fetal immune cell profile in chimeras only from mIUT ($n = 16$) and pIUT ($n = 24$) recipient PB and BM combined. Individual values are represented on the graphs. A, mIUT chimeras had higher trafficked maternal CD19 at 8 pw (mean 58.23% vs 8.68%, $P < .0001$, Figure 3E), and (B) higher fetal CD19 at 8 pw (mean 63.21% vs 33.62%, $P < .0001$) and 12 pw (mean 75.73% vs 36.97%, $P = .02$) than observed in pIUT recipients. C, pIUT chimeras (denoted by C at the base of the bar graphs) showed higher percentage of maternal CD3 and CD8 cells, and lower CD19 cells, compared to non-chimeras (denoted by N at the base of the bar graphs) in PB and BM, and (D) higher percentage of fetal CD3, CD4, and CD19 in PB and BM than non-chimeras.

E, Maternal CD19 percentage levels were higher in mIUT chimeras (all organs combined) compared to non-chimeras, as were (F) fetal CD3 and CD19 levels. Multiple t test, $\alpha = 0.05$, values expressed as mean ± SD. PB: peripheral blood, BM: bone marrow.

Serum harvested from non-chimeric offspring postnatally boosted with donor cells were analyzed for donor-specific IgM and IgG (Figure 6E). Controls were provided by sensitized sera from B6 or BALB/c adult mice injected intraperitoneally with BALB/c or B6 splenocytes, respectively, and naïve sera collected from age-matched crossbred F1 pups from uninterrupted pregnancies. Donor-specific IgM and IgG were expressed by 1.4% and 2.7% of non-chimeric mIUT and pIUT pups, respectively (not significant). Both groups showed lower IgM and IgG responses before and after boosting compared to sensitized sera and similar to naïve controls (Figure 6F,G). Ig levels were negligible by 10 pw in IUT-recipient serum.

**3.6 | Maternal IUT recipients demonstrate greater T cell reactivity to donor and non-donor cells while paternal IUT recipients were relatively hyporesponsive**

We studied T cell proliferation of IUT-recipient splenocytes in a mixed lymphocyte reaction assay (MLR) in which unstimulated recipient splenocytes and stimulated
 naïve splenocytes from uninjected cross-bred F1 offspring served as controls. IUT-recipient splenocytes at 4 pw were harvested and cultured with mitotically inactivated B6 or BALB/c splenocytes for 72 hours. We chose 4 pw time point as the fetal splenocytes are mature enough to react to the presence of parental cells, and maternal trafficking cells are still in circulation. mIUT splenocytes stimulated with mitotically inactivated donor B6 splenocytes demonstrated a small but significant upregulation of CD8 Treg, CD4 and CD8 T-EM, and Teff compared with unstimulated recipient cells. mIUT splenocytes stimulated with non-donor BALB/c splenocytes produced higher CD4+CD25+Treg, CD4+ and CD8+CD62L+CD25+cells, CD4 T-SCM and T-CM, and CD4 and CD8 Teff cells compared to B6-stimulated and

FIGURE 6  Assessment of donor-specific tolerance. Percentage of MMc immune profile in (A) mIUT (n = 18) and (B) pIUT recipients (n = 12) following postnatal boost with donor cells (at 4 pw, 5E+6 cells/pup, solid symbols); controls were injected with saline (open symbols, n = 23). Levels of CD4, CD8, CD11c, CD19, and NK1.1 cells were compared in fetal organs at monthly intervals. Similarly fetal immune profiles of (C) mIUT and (D) pIUT recipients were also compared. No significant differences in MMc and fetal cells were found between boosted recipients and controls. E. Detection of donor-specific IgG and IgM antibodies in boosted recipients. Sera were collected and analyzed from treated offspring before and 2 weeks after postnatal cell boost. Assay standards were provided by sera collected from sensitized adult B6 mice injected with BALB/c splenocytes (shown in E) and BALB/c adults sensitized with IP injections of B6 splenocytes. Naïve sera were collected from age-matched cross-bred pups from uninterrupted pregnancies. (F) mIUT (n = 10) and (G) pIUT sera (n = 10) collected from recipients showed no differences in IgM or IgG levels compared with naïve sera, lower than sensitized sera at 4, 7, and 10 pw. IgM and IgG were undetectable by 10 pw in IUT recipients.
unstimulated cells (Figure 7A). PlUT splenocytes stimulated with donor BALB/c splenocytes showed significant upregulation of CD4 and CD8 CD25+Treg, CD4 T-SCM and T-EM; stimulation with non-donor B6 splenocytes upregulated CD8 CD25+Treg and CD62L+CD25+ cells, and upregulated CD4 and CD8 T-SCM, CD8 T-EM, and T-EM compared to unstimulated controls (Figure 7B). Thus, mIUT and pIUT recipients demonstrated Treg and memory T cell proliferation in response to both maternal and paternal donor cells. While mIUT recipients upregulated Teff upon exposure to non-donor BALB/c cells, pIUT recipient splenocytes showed similar reactions to both donor BALB/c and non-donor B6 stimulation.

Compared to naïve stimulated splenocytes, mIUT splenocytes demonstrated a relative downregulation of CD8 regulatory and memory T cells (Treg, CD62L+CD25+ cells, T-SCM, T-CM, and T-EM) in the presence of donor B6 and non-donor BALB/c splenocytes; there was relative proliferation of CD8 Teff following non-donor BALB/c stimulation. mIUT splenocytes showed greater reactivity to non-donor BALB/c cells than to donor B6 cells with greater proliferation of CD4 and CD8 Teff, in addition to regulatory CD4 and CD8 CD62L+CD25+ cells (summarized in Circos plot Figure 7C).24 Compared to naïve splenocytes, PlUT splenocytes demonstrated significant proliferation of CD4 and CD8 CD25+Treg, T-SCM, and T-EM, and downregulated CD8 Treg and CD62L+CD25+ cells when stimulated with donor BALB/c splenocytes. Stimulation with non-donor B6 splenocytes increased proliferation of CD8 CD25+Treg and CD62L+CD25+ cells, and CD4 and CD8 T-SCM, T-CM, and T-EM. Responsiveness to donor and non-donor cells was similar. These data suggest that mIUT recipients are more immune-reactive than naïve offspring (no intrauterine exposure), while pIUT recipients are more immune-suppressive.

**FIGURE 7** Cellular immune responses of IUT recipients in mixed lymphocyte reactivity (MLR) assays. A, Fold change in T cell profile of mIUT recipients (n = 6) was analyzed after 72 hours stimulation with mitomycin C-inactivated B6 or BALB/c splenocytes compared to unstimulated recipient splenocytes (control, horizontal dotted line) by FACS. BALB/c stimulation resulted in significantly higher CD4 and CD8 Treg, memory, and effector T cells over control; B6 stimulation generally did not produce significant change over controls, and these responses were significantly lower than responses to non-donor BALB/c cells. B, PlUT splenocytes (n = 6) showed enhanced proliferation of CD4 and CD8 Treg, memory, and effector T cells following stimulation with both BALB/c and B6 stimulation; there were no differences in responses to either donor or non-donor cells. C, This Circos plot visually represents the relative responsiveness of mIUT and PlUT splenocytes when stimulated with B6 or BALB/c cells compared to naïve (uninjected) splenocytes of Figure 2C. mIUT splenocytes upregulated CD4 and CD8 CD62L+CD25+Treg subset and CD4 and CD8 effector T cells when stimulated with BALB/c cells more than with B6. PlUT splenocytes produced similar responses to either donor cell, and upregulated CD8 Treg subsets following both B6 and BALB/c stimulation compared to naïve splenocytes. In contrast, mIUT splenocytes showed lower proliferation of CD8 Treg and upregulation of CD8 effector T cells when stimulated with either parental cell over naïve controls. * above the individual bar represents significant change from the control, while ** represents significant difference between B6 and BALB/c outcomes. Multiple t test and two-way ANOVA with Tukey’s correction, α = 0.05
The rare MMc cells harvested from IUT recipients did not show changes in T cell proliferation compared to controls in MLR assays (Supplementary Figure S1C).

### 3.7 Maternal IUT upregulated pro-inflammatory cytokines while paternal IUT recipients were hyporesponsive to donor and non-donor cells

Compared with unstimulated F1 splenocytes, mIUT recipients showed significant upregulation of most pro-inflammatory/pro-immune and immune-suppressive cytokines and proteins when stimulated with both donor and non-donor cells (Figure 8A). pIUT splenocytes produced elevated pro-inflammatory cytokines for example, IL2 and TNF-α and downregulated immune-suppressive IL17b, IL27, and TGFβ below the level of unstimulated splenocytes, particularly when exposed to donor BALB/c splenocytes (Figure 8A). Categorizing cytokines by their T cell associations, we observed a more prominent response by mIUT recipient splenocytes, demonstrating a profile associated with cellular and humoral immune responses (Th1, Th2, Th1/Th2, and Thfh) and inflammation (Th17 and Th22) when stimulated with both donor and non-donor splenocytes. The responsiveness from pIUT splenocytes was subdued by comparison, especially in Th2, Th17, and Th22 with both donor and non-donor cells (Figure 8B). We then compared the responsiveness of mIUT and pIUT recipients to donor and non-donor BALB/c splenocytes (Figure 8C). The Circos plot displays the relatively greater responsiveness of mIUT recipients when exposed to either B6 or BALB/c splenocytes compared to pIUT recipients. mIUT recipients produced higher fold changes in proimmune IL2, CSF2, IFNγ, TNFα, and immune-suppressive FOXP3 and TGFβ with donor B6 stimulation than pIUT recipients showed with donor BALB/c stimulation. mIUT splenocytes showed greater response to BALB/c cells than pIUT splenocytes, with higher fold change in proimmune IL2, CSF2, TNFα, and immune-suppressive FOXP3. In contrast, pIUT splenocytes showed similar cytokine activity to both B6 and BALB/c cells. * above the individual bar represents significant change from the control, while ** represents significant difference between B6 and BALB/c outcomes.

**FIGURE 8** Cytokine gene expression studies from MLR assays were analyzed by qPCR. A, Cytokine expression in mIUT (n = 6) and pIUT recipients (n = 6) exposed to B6 or BALB/c splenocytes. mIUT recipients showed significant upregulation of most pro-inflammatory/pro-immune and immune-suppressive cytokines with B6 and BALB/c stimulation compared to unstimulated recipient splenocytes (controls, horizontal dotted line), showing higher IL1b, IL1RA, IL21, and L12a with B6 than with BALB/c splenocytes. In contrast, while pIUT recipients upregulated most pro-inflammatory/pro-immune and immunosuppressive cytokines with B6 or BALB/c cells, there was a more subdued response overall. Values are expressed as fold change over unstimulated controls after normalizing to housekeeping gene GAPDH. Expression of IL-5 and IL-22 levels in pIUT samples were inconclusive. B, Cytokine expression classified by functional T cell activity. mIUT recipients demonstrated elevated production of cytokines associated with functional T cells mediating cell and humoral immune responses (Th1, Th2, Th1/Th2, and Thfh) and inflammation (Th17 and Th22) than pIUT. C. The Circos plot displays the relatively greater responsiveness of mIUT recipients when exposed to either B6 or BALB/c splenocytes compared to pIUT recipients. mIUT recipients produced higher fold changes in proimmune IL2, CSF2, IFNγ, TNFα, and immune-suppressive FOXP3 and TGFβ with donor B6 stimulation than pIUT recipients showed with donor BALB/c stimulation. mIUT splenocytes showed greater response to BALB/c cells than pIUT splenocytes, with higher fold change in proimmune IL2, CSF2, TNFα, and immune-suppressive FOXP3. In contrast, pIUT splenocytes showed similar cytokine activity to both B6 and BALB/c cells. * above the individual bar represents significant change from the control, while ** represents significant difference between B6 and BALB/c outcomes.
mIUT and pIUT recipient splenocytes to their respective B6 and BALB/c donor cells. mIUT splenocytes stimulated with donor B6 cells showed significantly higher activity of pro-inflammatory/proliferative IL2, IL3, IL12a, IL21 IL27, CSF2, IFNγ, TNFα, and immune-suppressive IL10, IL16, FOXP3, and TGFβ (summarized in Circos plot Figure 8C), compared to pIUT recipients stimulated with donor BALB/c cells. There was higher expression of IL1b, IL12a, IL21, and IL1RA from mIUT splenocytes stimulated with donor B6, compared to stimulation of mIUT with non-donor BALB/c cells. pIUT splenocytes showed no differences in cytokine responsiveness with either B6 or BALB/c stimulation, and a lower expression of mostly pro-inflammatory cytokines than mIUT splenocytes stimulated with either donor or non-donor cells.

4 | DISCUSSION

We demonstrate that intrauterine transplantation of maternal or paternal semi-allogeneic BM cells induces donor-specific tolerance (DST) in recipients, influenced by the pattern and frequency of trafficked maternal cells and the responsiveness of recipient immune cells. This is evident following both IUT and postnatal re-exposure to donor cells where chimeras demonstrate differences in maternal and fetal CD3, CD4, CD8, and CD19. Intrauterine exposure to paternal donor cells induces a more subdued cell-mediated immune responsiveness in the recipient, leading to persistent DCC. There are valuable clinical implications in these findings, as the biological father can serve as the transplant donor for IUT instead of the pregnant mother. Paternal HSPC can be collected by PB mobilization (not performed in pregnant women), and stressful pregnant mother. Paternal HSPC can be collected by PB mobilization (not performed in pregnant women), and stressful

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difficulties in human IUT. This mixed-breed technical model allows parallel study of trafficked maternal immune cells and transplanted unmarked maternal donor cells in the recipient due to the use of cosogenic breeds of B6 females, as we begin to delineate the complex interactions between the parental donor cell exposure, maternal cell influx, and the fetal immune response. We used uniform intrauterine doses of 10E+9 cells/kg per pup (0.5 g) and 4.2E+8 cells/kg per pup postnatally (4w, 12 g). We did not use a high inoculum dose but still observed 75% chimerism frequency in pIUT recipients. Our initial selection for LSK cells yielded a substantially depleted cell pool insufficient to inject entire multifetal litters, as the LSK population represents about 0.1% of our initial BM MNC, similar to other reports. Thus we transplanted BM MNC which maximized cell yield with the beneficial inclusion of short- and long-term repopulating HSPC. We observed higher engraftment (3.0%-3.6% among chimeras) to levels previously reported for the same dose delivered IH at E14. Maternal and paternal donor cells did not differ apart from Sca1, which is poorly expressed on BALB/c cells, and maintained multilineage engraftment. As donor cells are semi-allogeneic to all F1 recipients, and as all injections were performed consistently by the same investigators, the observed responses are likely related to recipient immune response to the donor cells used. Large data variations were observed at various time points despite our efforts to minimize inter-experimental differences in IUT and handling of fragile tissues particularly from E12 and E18 fetuses, and partly related to the relatively small sample sizes at certain time points.

Unlike a previous murine study in which MMc was observed only in treated pups via IUT-associated hepatic injury, and not in naïve mice, we observed trafficked maternal cells in multiple organs of naïve animals at a lower frequency than in injected fetuses. The different maternal trafficking patterns in treated and untreated mice, and in chimeras and non-chimeras, are reflective of different mechanisms such as increased maternal T cell recruitment, selective proliferation, increased homing of maternal cells to fetal organs. The maternal immune response is a formidable engraftment barrier acting via donor-specific alloantibodies or effector T cells, inducing a parallel adaptive immune response in the recipient causing early or late transplant rejection. Though increased maternal-fetal T cell trafficking accompanying IUT been reported as the cause of graft rejection and pregnancy loss, other reports suggest that T cell influx at transplantation releases inhibitory molecules, attracts an influx of anti-inflammatory APCs, reduces conventional T cell activation, induces FOXP3+Treg production via IL2 and TGFβ expression, and contributes to epigenetic silencing of important chemokines. The elevated trafficking of maternal CD3, CD4, and CD19 cells in our hybrid chimeras may reflect increased transfer of regulatory cells to the recipient fetus, although we were unable to characterize these further due to the scarcity of these cells (insufficient for functional studies). The upregulated Tregs and the high expression of anti-inflammatory cytokines seen upon stimulation with donor cells in our study may have the effect of increasing the threshold for T cell activation. This correlates with recently published data describing injection of host-derived traditional Treg to promote allo-engraftment in immunocompetent mice.

Another putative mechanism is the generation of regulatory B cells (Breg) which have recently emerged as important mediators of transplant success. Though we did not find evidence of a donor-specific antibody-mediated response, this does not exclude Breg responses acting via IL10 and TGFβ. Breg markers are heterogeneous and non-specific so we did not further characterize CD19 cells; we did observe that chimeras from mIUT showed higher maternal and fetal CD19, while pIUT chimeras had lower maternal and fetal CD19. In mIUT recipients, IL10 and TGFβ were upregulated upon stimulation with both parental cells, while pIUT mice showed minimal changes in IL10 and TGFβ expression, except for the downregulation of TGFβ with BALB/c stimulation in keeping with the CD19 differences. We interpret from this that Breg response in the fetal-maternal immune milieu could be a possible mechanism through which mIUT recipients gain tolerance to donor cells; this will require further studies. Interestingly, our data showed contrasting NK findings. While mIUT non-chimeras demonstrated higher NK cell trafficking than mIUT chimeras, this was the reverse in pIUT where higher chimerism was associated with higher NK trafficking. We did not further characterize these NK cells but we posit that maternal NK cells are not necessarily detrimental to allografts.

Chimeric mice develop DST by 5 weeks post-transplantation when host and donor-derived APC mature, and have higher levels of suppressive Tregs which survive the thymic deletion of reactive effector T cells, resulting in an elevated proportion of recipient Treg after IUT. Chimeras also show decreased frequencies of alloreactive CD4+ and CD8+ T cells compared to naïve pups. Non-chimeras present insufficient antigen to allow complete recognition and deletion of effector T cells centrally, resulting in a relatively lower Treg effect and late-phase loss of engraftment via alloreactive T cells. This partly explains the lower chimerism frequency (40%-50% vs 80%) and eventual loss of engraftment reported in murine recipients of allogeneic compared to congenic IUT recipients. To further understand our findings, we considered the effects of exposure to inherit and non-inherited maternal and paternal antigens and associated mechanisms of fetal tolerance. The fetus is exposed to maternal and paternal antigens during development, setting in motion a series of events which serve to protect the fetal allograft from
maternal rejection and induce maternal tolerance in the fetus. Exposure to non-inherited maternal antigens (NIMA) via intrauterine seeding of maternal cells in to fetal LN induces peripheral development of fetal NIMA-specific CD4+CD25+FOXP3 Treg, which suppress the anti-NIMA behavior of fetal effector T cells via TGFβ, IL10, and IL35 expression.\textsuperscript{55,56} We found maternal CD4 and CD8 T, B and NK cells and occasional CD11c in several fetal tissues including LN; these cells may anergize NIMA-specific CD4 and CD8 effector T cells due to lack of co-stimulation and promote central clonal deletion contributing to overall fetal hyporesponsiveness.\textsuperscript{48,55} Even non-chimeric mice demonstrate durable T cell hyporesponsiveness and DST.\textsuperscript{45,57} We observed increased CD4+CD25+FOXP3 Treg and TGFβ expression in mIUT and pIUT recipients. Inherited paternal antigens (IPA) expressed by the fetus (Y chromosome-coded minor histocompatibility antigens, mHAg) elicit mHAg-specific class I-restricted CD8+suppressor T cells in both mother and offspring via the indirect allorecognition pathway.\textsuperscript{47,58} In our model of pIUT, the fetal and maternal immune systems are exposed to non-inherited paternal antigens (NIPA) in addition to IPA. Pre-IUT exposure to IPA may have primed the maternal immune system toward the regulatory pathway even prior to paternal cell exposure.\textsuperscript{59} We speculate that this double exposure to IPA and NIPA leads to rapid production of NIPA-specific maternal and fetal-induced Treg and mHAg-specific Treg using existing regulatory mechanisms. The resultant peripheral tolerance to NIMA (and NIPA) creates immunological niches in which paternal cells can survive.\textsuperscript{60,61} While we did not evaluate cytotoxic T lymphocyte function in this study, we observed lower CD8 effector T cell and IFNγ expression, and higher CD8 CD25+Treg and CD8 CD62L+CD25+ T cells in pIUT recipients compared with miUT mice. This together with the relatively lower expression of Th1-, Th2-, and Th17-associated cytokines in pIUT pups predicts higher maternal Treg activity and tolerance, important to establish allogeneic graft tolerance.\textsuperscript{48,59,62,63} The role of breastfeeding is interesting. While widespread intrauterine NIMA biodistribution results in early NIMA-specific tolerance in the fetus, continued postnatal exposure via breast milk encourages late NIMA-specific tolerance, reinforced by Treg and TGFβ transfer via breast milk which maintains long-term MMc.\textsuperscript{64,65} Yet, allogenic murine IUT resulted in loss of engrafted donor cells due to alloreactive maternal T cells and antibodies, avoidable with fostering.\textsuperscript{12,14} Our pups were not fostered to allow continued maternal immune transfer; despite this we did not detect donor cell-specific IgG or IgM nor substantial engraftment loss with pIUT. As with T and NK cells, the formation of DSA is likely antigen-dose-dependent, which we may have avoided using a low-dose inoculum. Our data also appear to contradict findings from HCT, in which the incidence of GVHD was lower with maternal or NIMA-mismatched graft than with paternal or NIPA-mismatched transplant.\textsuperscript{62,66} This study, like its predecessors, affirms that the most important advantage of IUT is the ability to prime the recipient for DST with early exposure, so that HCT can augment engraftment to achieve the desired therapeutic effect. This underlines the importance of IUT as an intervention for genetic conditions, even those that manifest postnatally such as the major β-hemoglobinopathies. Our studies were conducted on immune-competent mice, reflecting the majority of conditions in which IUT is potentially beneficial (eg, β-hemoglobinopathies) where normal maternal-fetal immune responses are expected. Combining the evidence from murine, canine, and NHP studies, we can recommend a therapeutic strategy for any perinatally significant genetic condition that starts with IUT during the physiological “window of opportunity.”\textsuperscript{29,67} using low-dose paternal BM or mobilized PB HSC to induce DST and educate the fetal immune response, followed by HCT from the same donor to achieve postnatal therapeutic engraftment, with minimal BM conditioning of the recipient if necessary. When this therapeutic strategy is applied in clinical trials, the main anticipated benefit is the avoidance of acute morbidity associated with the immunosuppression required when postnatal HSC transplantation is performed for example, for children with β-thalassemia major.\textsuperscript{68} 5  CONCLUSIONS In this direct comparison of maternal and paternal donor IUT and found the differentiating factors to be the origin of donor cells (NIMA vs NIPA), the pattern and persistence of MMc and fetal immune responsiveness. Fetal immune tolerance can be influenced by transplanting semi-allogeneic BM cells from either parent, but pIUT facilitates a more tolerant response especially to postnatal HCT. This carries several translational advantages, such as the reassurance of an alternative lifetime source of donor (paternal) HSC available for repeat postnatal transplantations. The slightly different maternal immune cell profile in naive and treated animals supports the concept of selective transplacental trafficking of maternal cells following IUT. ACKNOWLEDGMENTS This study was funded by the Singapore Ministry of Health National Medical Research Council NMRC/CSA-INV/0012/2016. CM is supported by grants from the NMRC/TA/0003/2012 and NMRC/CSA-INV/0012/2016. CONFLICT OF INTEREST The authors have declared that no conflict of interest exists.
AUTHOR CONTRIBUTIONS
K. Kandasamy, L.G. Tan, C.N.Z. Mattar designed and performed research, analyzed data and wrote the manuscript; N.B. Johana, Y.W. Tan, W. Foo, J.S.L. Yeo, V. Ravikumar performed the research, analyzed data and wrote the manuscript; F. Ginhoux, M. Choolani, J.K.Y. Chan assisted with data analysis and revised the manuscript. C.N.Z. Mattar supervised experimental design and manuscript preparation.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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