# Maternal Dendritic Cells Influence Fetal Allograft Response following Murine In-Utero Hematopoietic Stem Cell Transplantation

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# Abstract

Intrauterine hematopoietic cell transplantation (IUT), potentially curative in congenital haematological disease, is limited by subtherapeutic donor cell chimerism. Microchimerism of maternal immune cells trafficked into IUT recipients may directly influence donor-specific alloresponsiveness. We investigated if maternal dendritic cell (DC) depletion affected recipient alloresponsiveness and donor cell chimerism. IUT was performed on fetuses at embryonic day 14 (E-14), after crossing transgenic CD11c.DTR (C57BL/6) female mice and BALB/c male mice, with semi-allogenic BALB/c or C57BL/6, or fully allogenic C3H bone marrow donor cells, following transient maternal DC depletion with diphtheria toxin administered to the dam. We observed reduced maternally trafficked cells in the recipient fetuses particularly following BALB/c donor cell transplantation. IUT enriched trafficked maternal-derived clonotypes which displayed substantially reduced diversity. DC depletion restored maternal clonotype diversity and enhanced donor-specific immunomodulatory changes in the recipient pups, increasing regulatory T-cell subtype and immune-inhibitory protein expression, and inhibiting proinflammatory cytokine and donor-specific antibody production, though it did not affect donor cell chimerism. Functional tolerance acquired after BALB/c donor cell IUT with DC depletion was maintained following postnatal transplantation without immunosuppression, despite diminishing peripheral blood donor chimerism. We show for the first time that trafficked maternal cells influences donor-specific alloresponsiveness to IUT, possibly by expanding alloreactive clonotypes in the recipients, and depleting maternal DC promotes and maintains acquired tolerance independent of donor cell chimerism, presenting a novel approach to improve IUT efficacy.


# Introduction

*In utero* hematopoietic stem cell transplantation (IUHCT) has the potential to cure several congenital hematological disorders, with numerous advantages over conventional postnatal hematopoietic stem cell transplantation (HSCT), particularly the avoidance of myeloablation and immunosuppression [1]. Clinical application of IUHCT has however been hampered by poor engraftment due to numerous engraftment barriers, of which fetal and maternal immune responses to transplanted cells are formidable examples, leading to loss of donor cell chimerism (DCC) by immunological clearance [2–5]. Active trafficking of maternal immune cells (maternal microchimerism, MMc) to the fetus occurs throughout pregnancy, and can persist for years after birth [6, 7]. Substantial increases in trafficked maternal leukocytes and alloantibodies into recipient fetuses follow *in utero* transplantation of stem cells [8, 9], infusion of gene therapy vectors [10], and in response to the invasive procedure itself [11], which in turn limits donor cell engraftment. Previously we demonstrated the selective trafficking of maternal CD4, CD8, CD19 and CD11c immune cells into fetuses accompanying IUT, and haploidentical donor cells derived from paternal bone marrow engrafted more efficiently than maternal donor cells [8]. This was associated with a more regulatory T cell (Treg) and less pro-immune and pro-inflammatory recipient immune profile. Maternal and fetal dendritic cells (DC) are important to both innate and adaptive immunity, and, being the most important antigen-presenting cells for naïve T cells [12], mediate antigen-specific tolerance via altered expression of costimulatory molecules and cytokines [13–15]. We focus on myeloid conventional

dendritic cells (cDC) CD11c + CD123- DC subtypes cDC1 (XCR1+), which mediates efficient antigen recognition and cross-presentation to CD8 T cells via MHCI, and cDC2 which activates CD4 T cells via MHCII, as they are the most frequent DC populations in blood and lymphoid tissues and influence helper T cell responses [16–18]. DC can produce immunogenic or tolerogenic responses by altering the balance of Th1/Th17/Th2 cells as dictated by the specific microenvironment, and shift the immune milieu towards autoimmune and cytotoxic responses, or peripheral tolerance [19, 20]. DC are also involved in controlling inherent T cell autoreactivity, contributing to central T cell tolerance [21], and play a critical role in the generation of Treg that suppress effector T cell responses [22, 23]. Human DC migration commences in mid-gestation and fetal immune cells are immunologically-responsive, and may be influenced by trafficked maternal DC in response to *in utero* transplantation [24, 25]. Thus, transient suppression of maternal DC at the time of IUT may enhance chimerism by allowing donor cells to bypass initial antigen recognition and subsequent T cell activation in recipients. Here, we investigate if long-term engraftment of semi-allogenic and fully allogenic donor cells is influenced by transient maternal DC suppression in a transgenic mouse model of IUT. We interrogated the functional profile, gene expression and T cell and B cell receptor repertoires of trafficked maternal immune cells and the IUT recipient's immune response to donor cells. We studied this from the perspective of paternal donor cell IUT (pIUT) as this was the most efficient transplantation strategy from our previous study [8], and compared outcomes with maternal donor cell IUT (mIUT) and allogenic donor cell IUT (aIUT).

# Results

### Maternal CD11c+MHC-II+ DC depletion influenced trafficked maternal cells following IUT.

Diphtheria toxin (DT) administration to non-pregnant CD11c-DTR females depleted CD11c+MHC-II+cDC in spleen, bone marrow (BM), peripheral blood (PB) and uterus (**Supplemental data Figure S1a**) [26]. Baseline cDC (0.23-0.38%) reached troughs (0.01-0.06%) 2-7 days post-DT, returning to baseline on day 7 in BM and uterus (**Figure 1a**). cDC1 was <0.1% at all timepoints, while cDC2 was returned to 0.86% in BM by day 7. Other immune cells remained unaffected. Our IUT model (CD11c-DTR females crossed with BALB/c males) allowed us to track donor, maternal and recipient cells separately **(Supplemental data Figure S1b)**. Intrahepatic IUT utilising donor cells from maternal (B6 mice, referred to as mIUT), paternal (BALB/c mice, referred to as pIUT) or allogenic (C3H mice, referred to as aIUT) BM-MNC produced survival rates in DT- (controls, injected with saline) and DT+ (transient DC suppression, injected with DT) pups respectively of 92.0% and 76.9% (non-IUT controls, n=61), 75.0% and 79.6% (pIUT, n=60), 81.1% and 45.0% (mIUT, n=31), 100.0% and 46.7% (aIUT, n=22, **Figure 1b**).

Next, we determined that microchimeric maternal cells (MMc) following DT+pIUT and DT-pIUT were similar in BM (0.32-0.48%) and PB (0.43-0.78%), higher after DT+mIUT in BM than PB (4-8w, $p<0.05$, **Figure 1c**), and higher in DT-aIUT than DT-pIUT (8w, $p<0.05$, **Figure 1c,d**). cDC was observed only in DT-IUT BM (0.03-0.08%), and in DT+aIUT and DT-aIUT PB (0.04-0.08%); cDC1 and cDC2 were detected only in DT-mIUT and DT-aIUT BM (0.03-0.04%, **Figure 1d**). Compared to DT-, we observed non-significant reductions in CD4, CD8, CD19 in DT+pIUT and DT+mIUT BM, and higher CD4, CD8 in DT+aIUT BM (**Figure 1d**), with

non-significant increases in CD4, CD8 in DT+pIUT and DT+mIUT PB, and reductions in all cells in DT+aIUT, particularly NK1.1 (10.4±10.4% v 29.9±10.7%, p<0.0001). Thus, maternal DC depletion influences MMc differently depending on IUT.

### Donor cell chimerism was highest following pIUT and hematopoietic markers were retained

We observed higher donor cell chimerism (DCC) in BM ($DCC^{BM}$) in DT-pIUT than in DT-mIUT and DT-aIUT recipients (0w, p<0.05), and higher DCC in PB ($DCC^{PB}$) in DT-pIUT compared to DT-aIUT (4-12w, p<0.05). DT-mIUT showed higher $DCC^{PB}$ than DT-aIUT and DT+mIUT (0w, p<0.05). With DT+pIUT, $DCC^{BM}$ was maintained >2% until 12w, though $DCC^{PB}$ declined to <1% by 8w ($DCC^{BM}$ v $DCC^{PB}$ at 12w, p<0.001, **Figure 1c**). $DCC^{BM}$ in DT-mIUT and DT+mIUT remained <1% while $DCC^{PB}$ declined rapidly from 11.07±0.07% (0w, DT-mIUT), and remained <1% (0-12w, DT+mIUT). $DCC^{BM}$ and $DCC^{PB}$ in DT-aIUT and DT+aIUT remained <1% (0-12w). Recovered $DCC^{BM}$ and $DCC^{PB}$ showed similar proportions of Lin-Sca1+c-Kit+ (LSK), CD48+ and CD150+ indicating the presence of long-term repopulating HSC (**Supplemental data Figure S1c**). We deduced that post-IUT maternal cell trafficking was an active process, as we observed significant reductions in CD3 and CD19 in recipient BM and PB compared to uninjected dams (all differences, p<0.0001), except for NK1.1 which was higher in aIUT recipients than controls (p<0.0001, **Supplemental data Figure S2a**). Thus, DCC appears to be independent of MMc.

### Maternal DC depletion increased recipient Treg and reduced cytokine expression associated with alloreactivity.

We next examined the impact of maternal DC depletion on DCC and alloresponsiveness of IUT recipients. In DT+pIUT, CD19 was significantly higher in $DCC^{BM}$ (4-8w, p<0.05) and in $DCC^{PB}$ (0-12w, p<0.05) than other cells. In DT-$DCC^{PB}$, CD19 was more prevalent at 0w (p<0.0001, **Figure 2a, b**). Within corresponding MMc, maternal CD3 was significantly lower in DT+pIUT compared to DT-pIUT (where it is the most prevalent cell) at 0-12w (p<0.005) and was also higher than cDC subsets in DT+pIUT (4w, p<0.05) (data not shown); no other differences were observed in MMc between groups (**Figure 2c, d**). DT+pIUT and DT-pIUT produced higher CD19 than other immune cells in BM and PB (p<0.005 at all timepoints, **Figure 2e,f**) with no differences between groups.

In vitro assessment of functional tolerance was performed by MLR at 8w when pIUT recipients had waning $DCC^{PB}$ at <1% though $DCC^{BM}$ was maintained. Here, re-exposure of DT+pIUT splenocytes to BALB/c cells elicited greater expression of CD4 effector memory (Tm), regulatory (Treg) and CD25+Treg compared to DT-pIUT and untreated controls (p<0.001, **Figure 2g**), and re-exposure to B6 cells produced higher CD4 Treg compared to DT-pIUT and controls (p<0.001). DT+pIUT recipients showed greater fold-change in CD8 central Tm, effector (Teff), Treg and CD62L+CD25+Treg (p<0.001) when challenged with B6 cells, compared with controls and DT- recipients (**Figure 2h**). DT-pIUT produced greater fold-changes in cytokines associated with helper T-cells,[27] when stimulated with BALB/c or B6 cells, while a relatively lower response was observed for DT+pIUT (**Figure 2i-n**).

Though $DCC^{BM}$ and $DCC^{PB}$ were <0.2% in DT+mIUT, we were able to analyse donor immune cell components **(Figure 3a,b**). $DCC^{BM}$ in DT+mIUT contained higher CD8, CD19 than cDC, NK1.1 ($p<0.05$), and compared to DT-mIUT, $DCC^{PB}$ showed lower CD8 ($p<0.05$, **Figure 3b**). There were no differences in MMc (**Figure 3c,d**) and recipient immune profile (**Figure 3e,f**) in DT+mIUT BM or PB compared to DT-mIUT.

DT+mIUT elevated CD4 effector Tm, CD4 and CD8 Teff, and CD4 and CD8 CD25+Treg, CD62L+CD25+Treg when stimulated with BALB/c cells only on MLR (**Figure 3g, h**); only low-level responses to B6 cells were elicited. Cytokine expression was much lower in mIUT than pIUT recipients (<6-fold increase over untreated controls). DT+mIUT produced a higher expression of largely inhibitory proteins (IL5, IL6, IL10, FOXP3, TGFβ2) in combinations associated with Th2, Th1/Th2, Th17 cells, following B6 and BALB/c stimulation (**Figure 3i-n**).

In contrast, DT+aIUT and DT-aIUT resulted in only microchimeric $DCC^{BM}$ and $DCC^{PB}$, with similar donor immune cell profiles. No differences in MMc, trafficked maternal immune cell profile or recipient immune cell profile were observed (**Figure 4a-f**). Significant fold-change increases in CD4 central and effector Tm, and CD4 and CD8 Teff, CD25+Treg and CD62L+CD25+Treg were observed in DT+aIUT on MLR (**Figure 4g,h**), which also significantly increased expression of all cytokines and regulatory proteins except FOXP3 when stimulated with C3H, compared to DT-aIUT, implying proimmune helper T cell enhancement. aIUT produced cytokine responses close to control levels when stimulated with B6 and BALB/c, except for IL-17a, IL-17f, IL-22, augmented with DT+aIUT (**Figure 4i-n**).

We attempted to evaluate regulatory B-cells (Breg) which lack unique phenotypic markers [28, 29]. pIUT and mIUT augmented IL10, IL5, IL6, TGF-β2, FOXP3 expression (**Figure 2j,m; Figure 3j,m**) suggesting the presence of activated Breg in response to IUT. There were no differences in CD19 post-transplantation (**Figure 2e,f; Figure 3e,f**). Thus, maternal DC depletion may promote a tolerogenic response through both Treg and Breg.

### IUT and DC depletion influence maternal and recipient T- and B-cell receptor repertoire diversity

To further parse maternal and recipient immune interactions, we analysed gene expression profile and T-cell (TCR) and B-cell (BCR) receptor repertoires in MNC isolated from pIUT and mIUT recipients. Top 3000 up- and down-regulated genes in each group – uninjected (DT-, n=17), DT-mIUT (n=7), DT-pIUT (n=5), DT+pIUT (n=5) – were identified by enrichment score (ES) >1.3. Within treatment groups, recipient and maternal cells shared 483-736 common genes (**Figure 5a**). With mIUT, 26/174 enriched recipient gene clusters and 25/171 maternal clusters represented RNA and protein metabolism, hemopoiesis and immune system development, also represented by 21/169 recipient clusters and 49/177 maternal clusters following pIUT. With DT+pIUT, 20/153 recipient clusters additionally represented T-cell regulation. Upregulated DT-pIUT and DT+pIUT maternal clusters represented mitogen-activated protein kinases (MAPK) cascades, T-cell activation, and immune system development. All groups shared 48 common

genes, of which 6/40 highly enriched clusters represented cytokine stimulus response, immune system regulation, B-cell mediated immunity and adaptive immune response (**Figure 5b**).

Compared to uninjected controls, retrieval frequency of maternal-derived TCR and BCR clonotypes increased from 0.02% to 2.04-3.13% following mIUT and pIUT respectively (**Table 1**), and these showed reduced diversity, with Hill numbers (order 1, exponential of Shannon-Wiener indices)[30] of 18-29, compared to 941 in controls. DT+pIUT decreased maternal clonotype retrieval by 17.3-fold and restored diversity towards baseline (Hill number of 447). In contrast, mIUT and pIUT recipient-derived clonotypes (retrieval frequency 0.02-0.15%) showed increased diversity (Hill numbers 528-3728 v 64 in controls). DT+pIUT reduced diversity in recipients (Hill numbers from 3728 to 1637).

Maternal- and recipient-derived TCR and BCR clonotypes were similar in uninjected pups; BCR immunoglobulin heavy (IGH), κ (IGK), λ (IGL) chains made up ~81% of maternal and 75% of recipient clonotypes (**Figure 5c**). Expansion of TCR β-chain (TRB) clonotypes and reduction in IGH were observed among maternal-derived clonotypes following mIUT and DT-pIUT. DT+pIUT produced further increases in TRB, TRA (α-chain), TRD (δ-chain), IGH, IGK relative to DT-pIUT. Among recipient-derived clonotypes, we observed expanded TRB, TRG (γ-chain), and reduced IGH, IGK following mIUT and pIUT, and DT+pIUT further reduced TRB and increased IGK and IGH.

Mostly higher-order (3+) clonotypes were encountered with IUT (**Figure 5d,e**). Top 20% of clonotypes (Quantile 1, Q1) were most abundant in uninjected controls, and clonotypes in Q2 to Q5 were more abundant in IUT recipients. Individual abundances of top 5 maternal-derived clonotypes in each group were expanded with IUT and diminished with DT+ (**Figure 5d**), while recipient-derived top 5 clonotypes were most abundant in uninjected pups (**Figure 5e**). We observed a large number of public maternal-derived clonotypes between DT+pIUT and controls (**Figure 5f**), and a substantial number of public recipient-derived clonotypes between mIUT, pIUT and DT+pIUT (**Figure 5g**).

Complementarity-determining region 3 (CDR3) incorporates the VDJ recombination junctions, accounting for most of the repertoire variation mediating specific antigen recognition [31]. CDR3 regions of naïve and antigen-experienced clonotypes are longer and shorter respectively, reflecting antigen-driven selection [32]. We found a significant shift towards longer CDR3 in maternal TRG (from mIUT) and TRA (DT-pIUT, DT+pIUT), and shorter CDR3 in maternal TRB (DT+pIUT), recipient TRA (mIUT), and maternal and recipient IGH (all groups) **(Figure 6)**. Analyses of gene segment usage to functionally characterize these clonotypes by hierarchical clustering indicate similarities in V/J-segment usage between pIUT, DT+pIUT and mIUT recipient-derived clonotypes and DT+pIUT maternal-derived clonotypes, where IGH, IGK, TRA, TRB V/J-segments, and TRG and TRD J-segments, were upregulated and TRA J-segments were downregulated. We also observed uniqueness in V/J-segment usage between uninjected control, mIUT and pIUT clonotypes (**Figure 7a**). Functionality of the top 20 shared clonotypes, assessed by comparing CDR3 amino acid sequences to protein databases using Tomtom [33], revealed motif enrichment corresponding to production of Ras GTPase activating proteins, MAPK substrates, and signal transduction molecules, among others (**Figure 7b**). These data show that both maternal and recipient

immune cells respond to donor cells and that only maternal TCR and BCR diversity is affected by maternal DC depletion.

### Postnatal transplantation following maternal DC depletion and IUT demonstrated sustained donor-specific hyporesponsiveness at 16w

DT+pIUT recipients were transplanted with either parental donor cell without immunosuppression to assess their functional tolerance in vivo. At 12w postnatal, these offspring had microchimeric $DCC^{PB}$ but maintained $DCC^{BM}$ >10.0% (**Figure 2a-d**). Postnatal challenge of maternal cells at 12w increased recipient CD3 and decreased CD19 relative to paternal cells and saline only in lymph nodes (**Supplemental data Figure S2b-f**), and no differences in other immune cells were observed at 16w, 4 weeks after transplantation. No donor-specific IgG or IgM antibodies were observed 4 weeks post-transplantation (**Supplemental data Figure S2g**).

## Discussion

To our knowledge, this is the first report of maternal immune cells “primed” on exposure to donor cells influencing recipient immune responses to IUT. Our current data support our previous observations that paternal donor cells engraft more efficiently, and that donor-specific tolerance is acquired from IUT of donor BM from either parent. With the additional maternal DC depletion prior to IUT, we further show that trafficked maternal immune cells influence host immune response to IUT. Actively trafficked maternal cells demonstrate reduced TCR and BCR diversity, suggesting the expansion of specific clonotypes following exposure to parental donor cells, while maternal DC depletion restored diversity, particularly of maternal T clonotypes. While recipient clonotype diversity was preserved with maternal DC depletion, offspring showed enhanced tolerogenic effects mediated through CD4 and CD8 Treg, inhibition of effector T cells and pro-immune cytokines, enhancement of immune-inhibitory cytokines and FOXP3, and absence of DSA. These were even observed in fully allogenic C3H IUT following maternal DC depletion. Additionally, we found that functional tolerance was maintained in DT + pIUT recipients with diminishing and low PB donor and maternal chimerism (< 1%) at the 12th week postnatal boost, whereas in our earlier work pIUT recipients of postnatal boost at 4w were chimeras (PB DCC > 1%). This data supports the findings of Chen et al that persistent DCC is irrelevant to the maintenance of donor-specific tolerance [34], and suggests that maternal DC suppression prolongs host tolerance acquired from IUT even in the absence of sustained $DCC^{PB}$.

While earlier murine IUT studies concluded that DCC facilitates initial tolerance induction by diminishing donor-specific T-cell and NK cell alloresponsiveness, postulated to occur through Treg augmentation or NK surface receptor downregulation in F1 progeny [34, 35] these models utilised completely allogenic transplantations, whereas our robust semi-allogenic model more accurately reflects clinical IUT for alpha-thalassaemia major using haploidentical maternal bone marrow HSC (clinical trial NCT02986698) and is the first to explore the role of trafficked maternal cells. Our study’s main limitations include the lack of skin grafting to interrogate acquired tolerance in IUT recipients; we instead performed postnatal boost

without immunosuppression for this functional assessment. Though we demonstrated donor-specific humoral and cellular hyporesponsiveness to donor cells of both parental origins, postnatal transplantation was only performed on pIUT recipients due to time and cost restrictions. We do not have MLR or TCR/BCR gene expression data from challenged animals. Postnatal transplantations in other IUT recipients and with larger cell doses would have provided useful comparison data regarding the functional effects of the immunomodulatory changes we describe. DCC and immune response may be strain-related [36, 37], hence performing IUT in hybrid offspring of B6 males and BALB/c females would have been informative. We previously reported that this breeding pattern did not yield differences in maternal microchimerism [8]. We did not explore the role of recipient NK cells in maintaining acquired tolerance as these cells undergo adaptation of their Ly49 receptors upon donor antigen exposure and influence DCC [36] though we did not observe differences in recipient NK levels following maternal DC suppression or postnatal challenge.

Inducing the appropriate balance of Th1/Th17/Th2, which influence allograft tolerance or rejection [27, 38], may be critical to improving IUT transplantation tolerance. Maternal TCR/BCR clonotypes displayed substantially reduced diversity post-IUT, restored with DC depletion, while recipient-derived TCR enrichment was unaffected. Our findings indicate that DCC and MMc are separate yet equally important determinants of IUT effectiveness. pIUT produced the highest and most persistent DCC. In contrast to other reported models using maternal donor cells [39, 40], we observed microchimerism post-mIUT despite reduced MMc, similar to our earlier work [8]. Thus, DCC appears primarily dependent on cell origin, not MMc. We demonstrated that maternal cell trafficking is an active process, the quality of which appears to influence the fetal recipient's immune response to donor cells. Taken together, pIUT resulted in both highest DCC and lowest MMc, and maternal DC depletion further dampened recipient alloresponsiveness through upregulated Treg, the possible presence of Breg, and downregulated proimmune Th cells. aIUT produced both poorest DCC and most robust alloresponsiveness, contrasting with data from other IUT models in which allogenic donor cells have a competitive advantage [41–45]. Although reduced MMc was associated with increased Treg and Breg cytokine expression, upregulated Teff and an overwhelmingly proimmune cytokine response were present, expediting allogeneic cell rejection. Maternal DC may also present donor antigen to recipient immune cells, influencing the quality of immune response. Enhanced Treg production following IUT, particularly of CD62L + CD25 + FOXP3 + Tregs, protects against graft-v-host disease [46, 47], and together with putative Breg (expressing IL10, IL5, IL6, FOXP3, TGF-β), probably influenced the resulting tolerogenic or immunogenic responses [28, 29]. Human transplantation data indicate that persistent recipient cells inhibit donor haematopoiesis and TCR reconstitution [48–50]. IUT adds an interesting dimension to this, wherein the maternal immune system is the other "recipient" interacting with donor cells, and both maternal and recipient immune systems may impact long-term engraftment and maintenance of tolerance.

Trafficked maternal TCR and BCR clonotypes showing reduced diversity, variable CDR3 lengths and V- and J-segment usage indicate dynamic acquisition of allo-specificity, further underscoring the "sensitization" of maternal cells to transplanted donor cells. Donor cells transplanted directly into the fetus may leak into maternal circulation and participate in maternal immune modelling, selecting and

expanding particular clonotypes for trafficking, similar to native fetal microchimerism.[51] The return of DT + maternal clonotypes to higher baseline diversity supports this postulation. Increased recipient TCR diversity confirms that treated fetuses mount antigenic responses even to haploidentical cells, and the diversity remains the same in the DT + recipients suggesting that the fetuses are less affected by the DT treatment to the pregnant mother. Clinically-poor responders to postnatal HSCT show lower TCR diversity, [31, 52, 53] thus recipient TCR repertoire may be a useful biomarker of transplant rejection.[54]

Our murine model permitted specific depletion of antigen-presenting cDC in pregnant mice, for which there are no published data. cDC depletion at E13 would have endured for the remainder of the pregnancy (parturition ~ E20), resulting in naïve maternal cDC trafficked to fetal recipients. Of particular interest is depletion of uterine DC, a unique subtype critical to pregnancy-related tolerance and rejection [12], capable of eliminating fetal cells, which concentrate in maternal circulation towards parturition,[51] while not rejecting the fetus [55, 56]. Allogenic fetal cells presented by uterine DC can prime maternal tolerance towards paternal antigens via expansion or elimination of alloreactive or regulatory lymphocytes [57–59]. Donor cells transplanted in mid-gestation likely leak into maternal circulation, prompting reciprocal trafficking of primed maternal cells. Indeed, with DC depletion, maternal TCR clonotypes showed markedly greater diversity, and pIUT and mIUT recipients expanded Treg subsets and upregulated less immunogenic cytokines. Further investigation into uterine DC may intriguingly reveal a unique target for in-vivo or ex-vivo modulation to enhance transplantation tolerance [14].

Our data raise the exciting possibility of utilising unique maternal and recipient TCR/BCR repertoires in multiple fashion for IUT, and by extension other cellular fetal therapies, by serving as biomarkers of engraftment and immune tolerance, as therapeutic targets to improve transplantation tolerance, e.g., by transducing regulatory sequences into high avidity clonotypes, and as prognosticators of transplantation outcomes. Robust graft tolerance required to maintain life-long engraftment carries several layers,[60] that, in combination, may even permit repeat transplantation, including limiting maturation of high-avidity alloreactive T-cells and expanding high-avidity Treg [61–63]. Selective expansion of regulatory TCR clonotypes with distinctly tolerogenic phenotypes may be an individualizable strategy through which to achieve such tolerance [64]. Bregs maintain immune tolerance, but are more difficult to identify due to the absence of unique markers [65, 66]. Though maternal and recipient TCR clonotypes underwent the greatest expansion, we observed an increase in recipient-derived BCR clonotypes along with upregulated Breg-associated cytokines. This suggests that putative regulatory BCR clonotypes are represented and may be valuable components of IUT tolerance. Our data may influence clinical management even in the short-term, supporting the transplantation of paternal donor cells in clinical trials or short-term maternal immune-suppression at the time of IUT, similar to immunotherapy for recurrent miscarriages [67]. Our work is limited by transplantation of a small donor inoculum and limited monitoring for 16 weeks, while pandemic restrictions curtailed our assessment of DT + mIUT and aIUT immune repertoires. Additionally, in the limited number of mIUT and aIUT recipients harvested, donor chimerism was very low which may affect accuracy of donor cell analyses. Transplantation of larger doses to achieve therapeutic DCC and a longer surveillance of maternal/recipient immune profiles in larger animals will be informative for future clinical translation.

# Methods

**Animal experiments**. Inbred strains BALB/c (CD45.2, H2K-d) and C3H/HeNTac (H2K-k referred to as C3H) were obtained from In Vivos (Singapore). C57BL/6 mice (CD45.1, H2K-b) and CD11c-DTR female mice (B6.FVB-1700016L21RikTg$^{(Itgax-DTR/EGFP)57Lan}$/J, CD45.2, H2K-b) were purchased from The Jackson Laboratory (Bar Habor, ME) and maintained in a specific pathogen-free facility at NUS. BALB/c males and CD11c-DTR females were time-mated for IUT experiments. The pregnant mice were randomly chosen for IUT and for downstream experiments, as the probability of getting pregnant dams in a group of breeding mice occur by chance.

**Transient DC suppression.** CD11c-DTR females were given a single intraperitoneal injection of DT (5ng/g of body weight) for transient DC suppression (indicated as DT+) and control CD11c-DTR mice were given saline (indicated as DT-) for IUT and non-IUT experiments. For analysing the levels of conventional DC (cDC), the females were harvested on day 0,1,2,4 and 7 days post injection, and BM, PB, spleen and uterine horn organs were analysed for CD4, CD8, CD19, NK1.1, CD11c, MHC-II, XCR1 and CD172a immune parameters by flow cytometry. Conventional DC (cDC) were identified by the CD11c + MHC-II + markers and cDC subpopulation cDC1 (XCR1+) and cDC2 (CD172a+) cells were identified similarly by flow cytometry.

**In-utero transplantation (IUT) and postnatal transplantation.** Fresh donor bone marrow mononuclear cells (BM-MNC) were prepared as previously described.[8] The day before IUT (E13), CD11c-DTR pregnant mothers (DT+) were given intraperitoneal DT injections (5ng/g body weight). DT- dams received of saline. IUT was performed on E14 and all fetuses received intrahepatic injection of 5E + 6 donor inoculum, as described previously.[8] Maternal donor cell IUT (mIUT) was performed using B6 BM-MNC (CD45.1, H-2K$^{b}$), BALB/c BM-MNC was administered in paternal donor cell IUT (pIUT) and C3H BM-MNC were used for complete allogenic donor cell IUT (aIUT). Uninjected controls were offspring of DT + or DT- dams which did not undergo intrauterine transplantation. After littering, pups were nursed by their mothers and weaned at 4 weeks. Fetal cells were harvested from F1 cross-bred hybrid pups sacrificed between postnatal weeks 1 to 12 to assess donor cell chimerism (DCC), trafficked maternal cell microchimerism (MMc) and fetal immunological responses in peripheral blood (PB) and BM. In selected DT + pIUT offspring, postnatal transplantation was performed with 5E + 6 donor cells at 12 postnatal weeks administered via retro-orbital route, without bone marrow ablation or immunosuppression. Serum and organs were harvested 4 weeks later.

**Isolation of MNC and FACS analysis.** MNC were isolated from various organs and approximately 1E + 6 cells/tube were stained for surface markers to differentiate trafficked maternal cells (H2K-b+), and fetal recipient cells (double positive H2K-b+, H2K-d+) as reported.[8] Donor cells were identified from fetal recipient cells by the expression of CD45.1+ (maternal donor cells), H2K-d+ (paternal donor cells), H2K-k+ (C3H donor cells) surface markers. Further, cells were stained for T cells (expressing CD3, CD4, CD8, NK1.1), B cells (CD19), dendritic cells (CD11c, MHC-II, XCR1, CD172a), HSC markers (Sca1, c-Kit, CD48, CD150), hematopoietic lineage markers (CD11b, GR1, TER119, CD3, CD19, B220). All antibody-stained

cells were analyzed using BD X-20 Fortessa™ flow cytometer (BD Biosciences, Franklin Lakes NJ, accessed at the NUS Life Science Institute), The list of antibodies used for flow cytometry were provided in supplemental data table (S1). Each antibody was validated with positive control samples as per manufacturer's instructions. All raw data files were compensated and analysed using FlowJo™ software (FlowJo LLC, Ashland OR). Percentage of donor cells and maternal trafficked cells were calculated from the total live MNC. Percentage of immune populations were calculated from the respective total population of MMc, donor cells and recipient cells and the percentage of HSC markers were calculated from the total donor cells.

**Mixed lymphocyte reactivity assay (MLR) for functional T cell activity**. Splenocytes (responder mononuclear cells) were isolated from 4–6 week old F1 cross-bred IUT and naive (from uninterrupted pregnancies) pups. Lymphoid tissue MNC from BALB/c, B6 or C3H mice, treated with mitomycin C (50µg/ml), served as stimulator cells. MLR was performed as reported.[8] Cells were harvested after 72h post culture and analysed for T cell subset surface markers (CD4, CD8, CD25, CD62L, CD44) and intracellular marker FOXP3 in the responder cells by flow cytometry; part of the cells was used for RNA extraction and cytokine gene expression analysis by RT-qPCR. Markers for phenotypes: central memory T cells (central Tm, CD44 + CD62L+), effector memory T cells (effector Tm, CD44+), effector T cells (Teff, CD25+), regulatory T cells (Treg, FOXP3+).

**Quantitative gene expression analysis.** Total RNA extraction and qPCR was performed as described previously.[8] List of qPCR primers used were provided in Supplemental data table S2.

**Bulk RNA Sequencing.** Trafficked maternal and recipient cells were isolated from the whole body of (includes primary and secondary lymphoid organs) 1-week-old F1 hybrid neonates, (groups consists of uninjected offspring (no IUT, DT-, n = 17), mIUT (DT-, n = 7), pIUT (DT-, n = 5) and DT + pIUT (n = 5)) by magnetic cell sorting (MACS), followed by FACS, using H2K-b and H2-Kd antibodies respectively. Isolated maternal trafficked cells from each neonate were not sufficient for RNA-seq analysis as individual samples. We pooled the samples from a group of neonates to generate sufficient number of cells for sorting and performed RNA-seq. Thus, we do not have replicates for RNAseq samples that were analysed, but each sample represents a certain number of harvested animals. We were unable to isolate sufficient samples from DT + mIUT and aIUT groups for analyses. Total RNA was isolated from the sorted cells using RNeasy micro kits (Qiagen) with on-column RNase-free DNase digestion and eluted carrier-free. Total RNA was quantified, and the quality assessed using Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA), before cDNA library preparation. Samples were sequenced on a flow cell using HiSeq 4000 system (Illumina, San Diego, CA), with a read length of 150bp and 100 million reads per sample. The raw reads were analyzed with the RNAseq pipeline from nf-core[68] using the reference genome (GRCm39) and gene annotation (M26) from GENCODE (https://www.gencodegenes.org/). The gene read count table generated by featureCount in the RNAseq pipeline was analysed using edgeR software.[69, 70] The dispersion value was set at 0.4 as recommended in edgeR for experimental setups with no replicates.

**Gene expression.** Using log of fold-change (logFC) data, we selected the most up- and down-regulated genes (1500 each, for a total of 3000) from each treatment group to organize using an online Venn diagram generator (http://bioinformatics.psb.ugent.be/webtools/Venn/) to single out common genes across all the groups. We analysed functions of enriched genes using the Database for Annotation, Visualisation and Integrated Discovery (DAVID v6.8, https://david.ncifcrf.gov/).[71, 72]

**TCR and BCR repertoire analyses.** Raw reads were analyzed by MIXCR performed with settings of analysis of random fragments, RNA starting material and using the provided Mus musculus dataset, all others remaining as default settings.[73, 74] Results from MIXCR were imported into VDJtools (https://github.com/mikessh/vdjtools) to plot the clonotypes in PlotQuantileStats.[75]

**Detection of donor-specific antibodies (DSA).** Serum collected from harvested pups and serum collected 4w after postnatal transplantation were used for DSA. For generating positive control sera, wild-type B6 mice were sensitized with maternal (B6/CD45.1), paternal (BALB/c) or allogenic (C3H) splenocytes at a dose of 2E + 7 cells, injected intra-peritoneally at an interval of 2 weeks. Sera was collected after 14 days. DSA assay was performed using respective splenocytes, as described previously.[8]

**Statistical analyses.** Continuous data were analysed using Analysis of variance (2way ANOVA) with Tukey's multiple comparisons test, with a single pooled variance and multiple t tests for comparisons of individual parameters. Statistical significance was determined at $\alpha = 5.0\%$. Values are expressed as mean ± standard error of the mean (SEM). 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, CA, www.graphpad.com). Error bars were not provided on Fig. 2a-f, Fig. 3a-f and Fig. 5a-f, as they may mask a clear view of the graph bars. Instead, respective raw data (mean ± SEM), were provided in supplemental data tables S3-5). All experimental data were collected and analysis were performed by separate team of staffs. Investigators were blinded for the current study. The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

# Declarations


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### Competing Interests

The authors have declared that no conflict of interest exists.

### Author Contributions:

KK, CNZM designed and performed research, analyzed data, and wrote the manuscript; NBJ, LGT, YT, JYSL, LZ, KJ performed research, analyzed data and wrote the manuscript, FG, MC, JKYC assisted with data analysis and revised the manuscript. CNZM supervised experimental design and manuscript preparation.

### Data Availability:

The datasets generated and/or analysed during this study are available from the corresponding author on reasonable request. Correspondence and requests for materials should be addressed to Dr. Citra NZ Mattar.

### Data repositories

The RNA sequencing data generated in this study have been deposited in the NCBI GEO (Gene Expression Omnibus) database under accession code # GSE195771.

### Ethics approval

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.

**Supplemental Data (tables and figures, list of abbreviations)** are available for this paper.

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# Tables

**Table 1: RNAseq analysis of trafficked maternal and recipient cells for TCR and BCR repertoire clonotypes**

| Sample | Total counts | Clonotype number | Mean clonotype frequency | Hill numbers (order 1)<br><br>*exponential of the Shannon-Weiner Index |
|---|---|---|---|---|
| **Trafficked maternal immune cells** | | | | |
| Uninjected pup | 348684 | 4429 | 2.26E-4 | 941 |
| mIUT recipient | 8376 | 49 | 2.04E-2 | 29 |
| pIUT recipient | 3749 | 32 | 3.13E-2 | 18 |
| DT+ pIUT recipient<br><br>(maternal DC suppression) | 5537 | 552 | 1.81E-3 | 447 |
| **Fetal immune cells** | | | | |
| Uninjected pup | 66848 | 148 | 6.76E-4 | 64 |
| mIUT recipient | 4043 | 666 | 1.50E-3 | 528 |
| pIUT recipient | 48175 | 5488 | 1.82E-4 | 3728 |
| DT+ pIUT recipient<br><br>(maternal DC suppression) | 18579 | 3064 | 3.26E-4 | 1637 |

# Figures

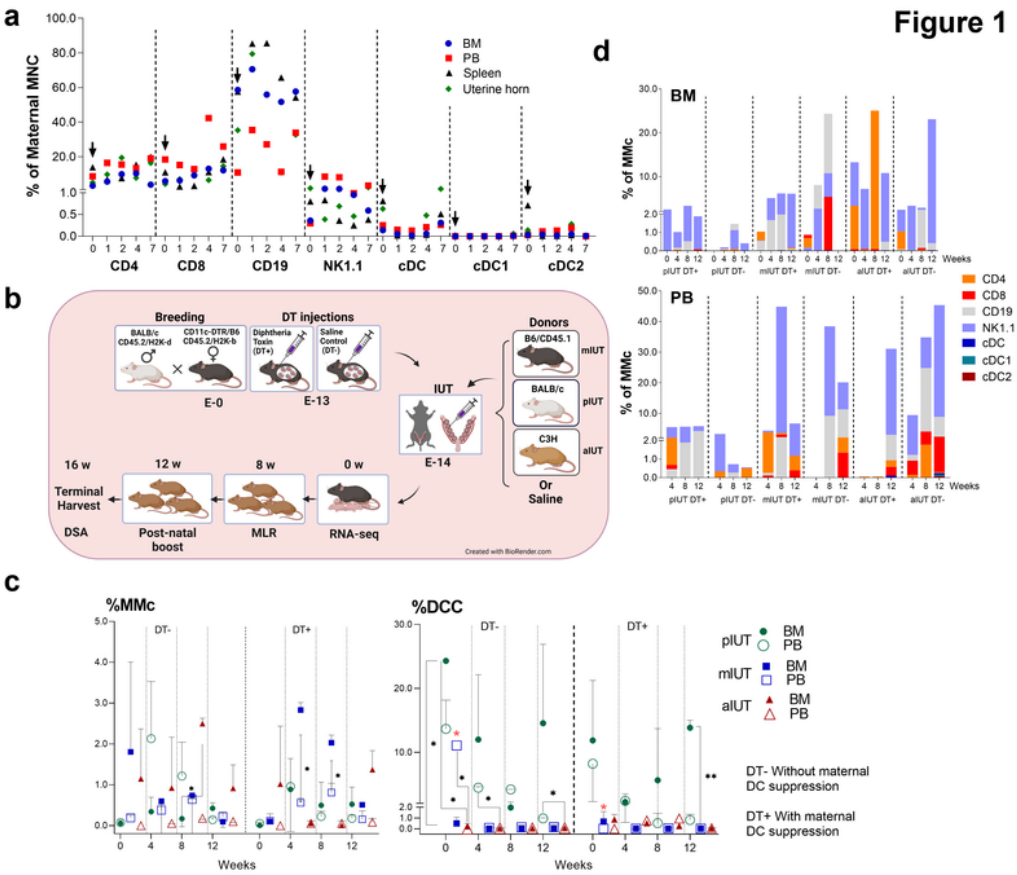


**Figure 1: Transient suppression of maternal dendritic cells with diphtheria toxin resulted in reduced maternal immune cell trafficking to fetal recipients following intrauterine transplantation of semi-allogenic and fully allogenic donor cells, and effect on donor cell chimerism.** Selective reduction of conventional dendritic cell (cDC) and subtypes (cDC1, cDC2) in various organs of non-pregnant CD11c.DTR females (n=5) after administering diphtheria toxin (DT, arrows, **a**). Intrauterine transplantations of maternal (n=31, mIUT) paternal (n=60, pIUT) and fully allogenic (n=22, aIUT) cells performed at E14 (**b**), with (DT+) or without DT (DT-). Maternal immune cell microchimerism (MMc) was similar in DT- and DT+ with each IUT (**c**), higher in aIUT v pIUT, and higher in DT+ bone marrow (BM) v peripheral blood (PB) after mIUT (black*). Donor cell chimerism (DCC) was highest with DT- pIUT in BM and PB (black*), and higher in mIUT PB v BM (red*). With DT+, DCC was maintained in pIUT BM (black*), and was higher in mIUT BM v PB (red*). Maternal cDC, CD4, CD19 cells were non-significantly reduced in DT+ recipient BM and higher in PB (**d**). cDC and subtypes were only found in DT-and DT+ aIUT recipients. Data represent mean±SEM, analysed by two-way ANOVA with Tukey's multiple comparisons test.

## Figure 1

See image above for figure legend

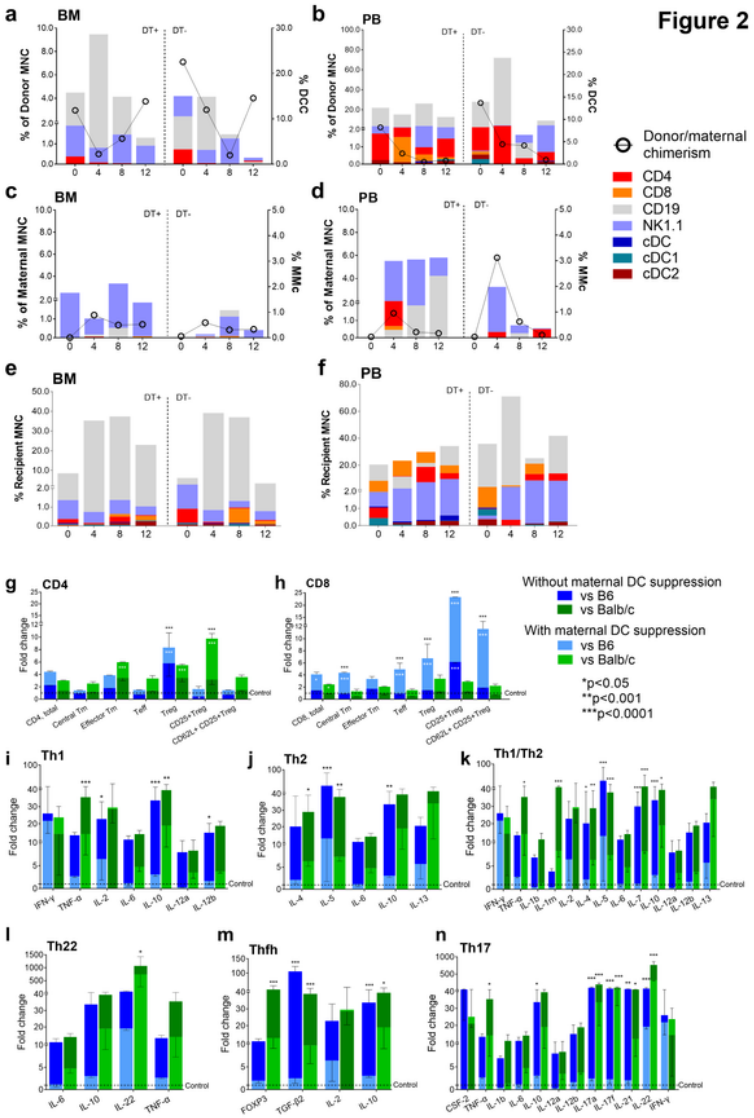


**Figure 2: Immune profiles of engrafted donor, trafficked maternal and recipient cells, T cell profile and cytokine, FOXP3 gene expression following paternal cell transplantation**. Compared to DT- (n=9), DT+ recipients (n=11) had engrafted donor cells showing reductions in all immune types relative to CD19 ($p<0.05$, **a,b**), and no significant changes in the trafficked maternal immune cells in bone marrow (BM) and peripheral blood (PB) **(c,d)**. Recipient immune cell profile was similar in DT+ and DT-, with relatively higher CD19 in both ($p<0.005$, **e,f**). Weeks shown on x-axis. DT+ (n=4) showed higher fold-changes in CD4+ and CD8+ (**g,h**) central and effector memory and regulatory T cells compared to uninjected controls (n=3, white*) and to DT- (n=3, black*). Reductions in helper T cell cytokines (**i-n**) were observed with DT+ (n=4) in response to paternal and maternal donor cells, compared to DT- (n=4). Data represent mean±SEM, analysed by two-way ANOVA with Tukey's multiple comparisons test.

## Figure 2

See image above for figure legend

**Figure 3: Immune profiles of engrafted donor, trafficked maternal and recipient cells, T cell profile and cytokine, FOXP3 gene expression following maternal cell transplantation.** Donor cell chimerism was low in DT+ (n=8) and DT- (n=7). In DT+, CD8, CD19 were higher in BM and CD8 lower in PB ($p<0.05$) than in DT- (a,b). MMc (c,d) and recipient immune cell profiles (e,f) were similar in DT+ and DT-. Weeks shown on x-axis. CD4+ effector memory, CD4+ and CD8+ effector and regulatory T cells in DT+ (n=4) were increased over DT- (n=3, black*) and over uninjected controls (n=3, white*) following paternal donor cell exposure (g,h). DT+ (n=4) and DT- (n=4) produced similar low-level responses in individual cytokines over untreated controls (n=2, black*) which were much lower than in paternal IUT (y-axis scale same as Figure 2i-n). DT+ produced higher expression of largely inhibitory cytokines associated with Th2, Th1/Th2, Th17 cells, following stimulation (i-n). Data represent mean±SEM, analysed by two-way ANOVA with Tukey's multiple comparisons test.

Figure 3

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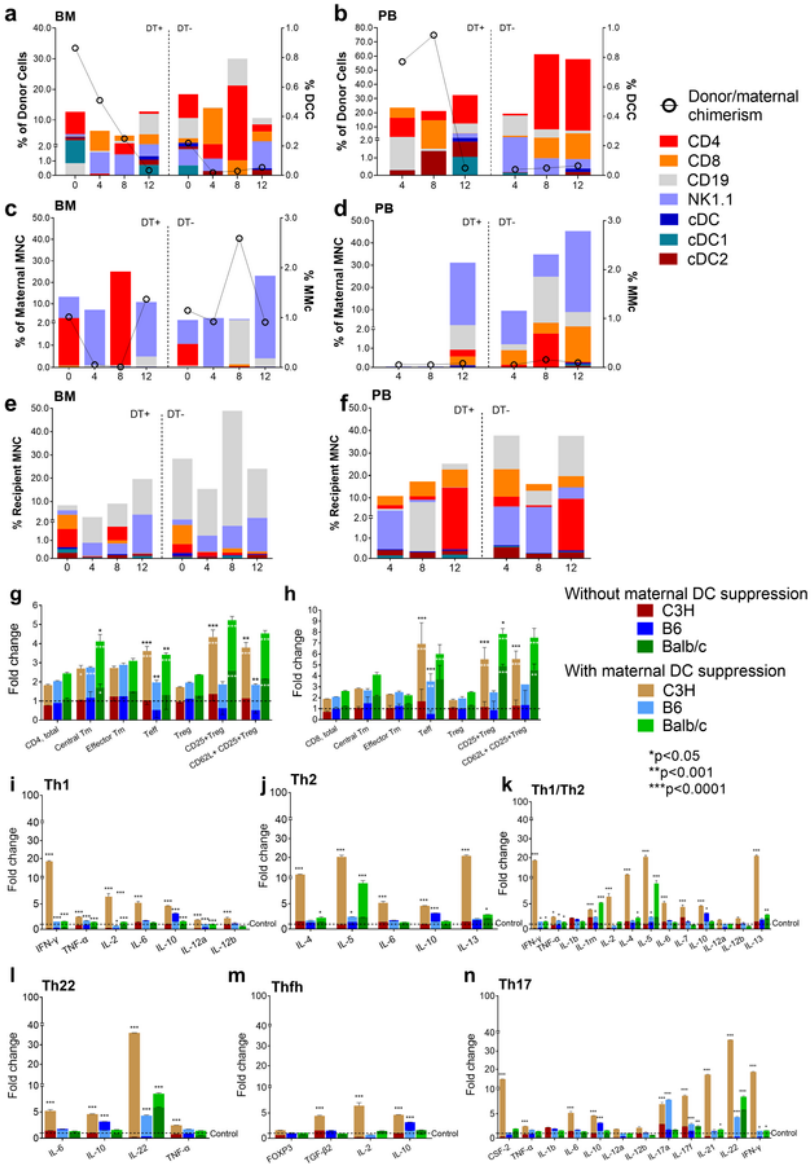


**Figure 4: Immune profiles of engrafted donor, trafficked maternal and recipient cells, T cell profile and cytokine, FOXP3 expression following allogenic cell transplantation.** Donor microchimerism <1% was observed in BM and PB (**a,b**). No differences in donor, maternal (**c,d**) or recipient (**e,f**) immune cell profiles were observed in DT+ and DT-. With DT+, CD4+ central and effector memory (Tm), CD4+ and CD8+ effector (Teff) and regulatory (Treg) T cells increased above DT- (black*) and uninjected controls (white*) on exposure to all donor cells (**g,h**), and increased all cytokines except FOXP3 with C3H exposure (**i-n**). Data represent mean ± SEM, analysed by two-way ANOVA with Tukey's multiple comparisons test.

## Figure 4

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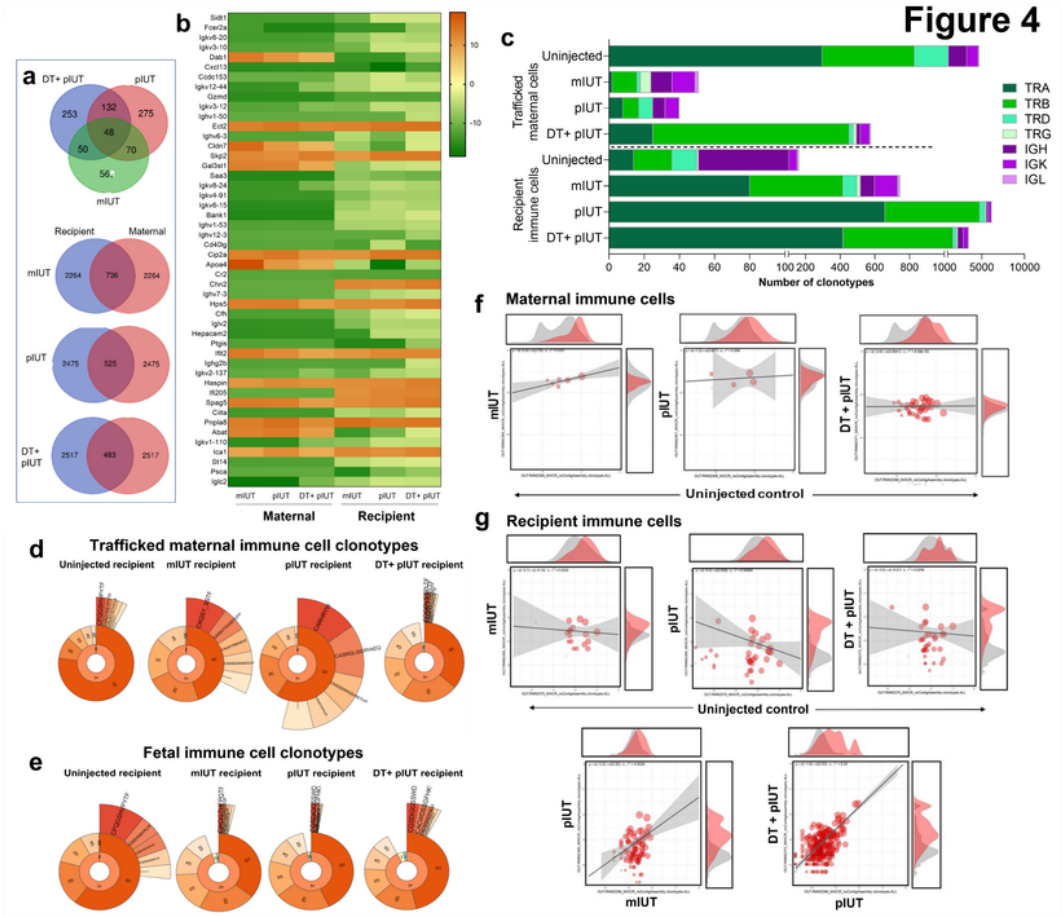


**Figure 5: Gene expression and T cell (TCR) and B cell (BCR) receptor repertoire of trafficked maternal and recipient immune cells.** Maternal and recipient immune cells from pIUT (DT+ n=5, DT- n=5), DT-mIUT (n=7) shared 48 genes (top panel) and up to 30% genes (bottom panel) within each IUT (**a**). Common gene clusters represent cell adhesion, cytokine response and immunoregulatory pathways (**b**). Maternal- and recipient-derived BCR and TCR clonotypes (**c**) were similar in uninjected pups (n=17); IUT increased TRB, TRG, reduced TRA, TRD, IGH, IGK in maternal-derived and recipient-derived clonotypes. Top 5 maternal-derived clonotypes were greatest following IUT and contracted in DT+pIUT (**d**). Recipient-derived top 5 clonotypes were most abundant in uninjected pups (**e**). Large overlaps observed between DT+pIUT and uninjected maternal-derived clonotypes (**f**) and between IUT recipients (**g**).

## Figure 5

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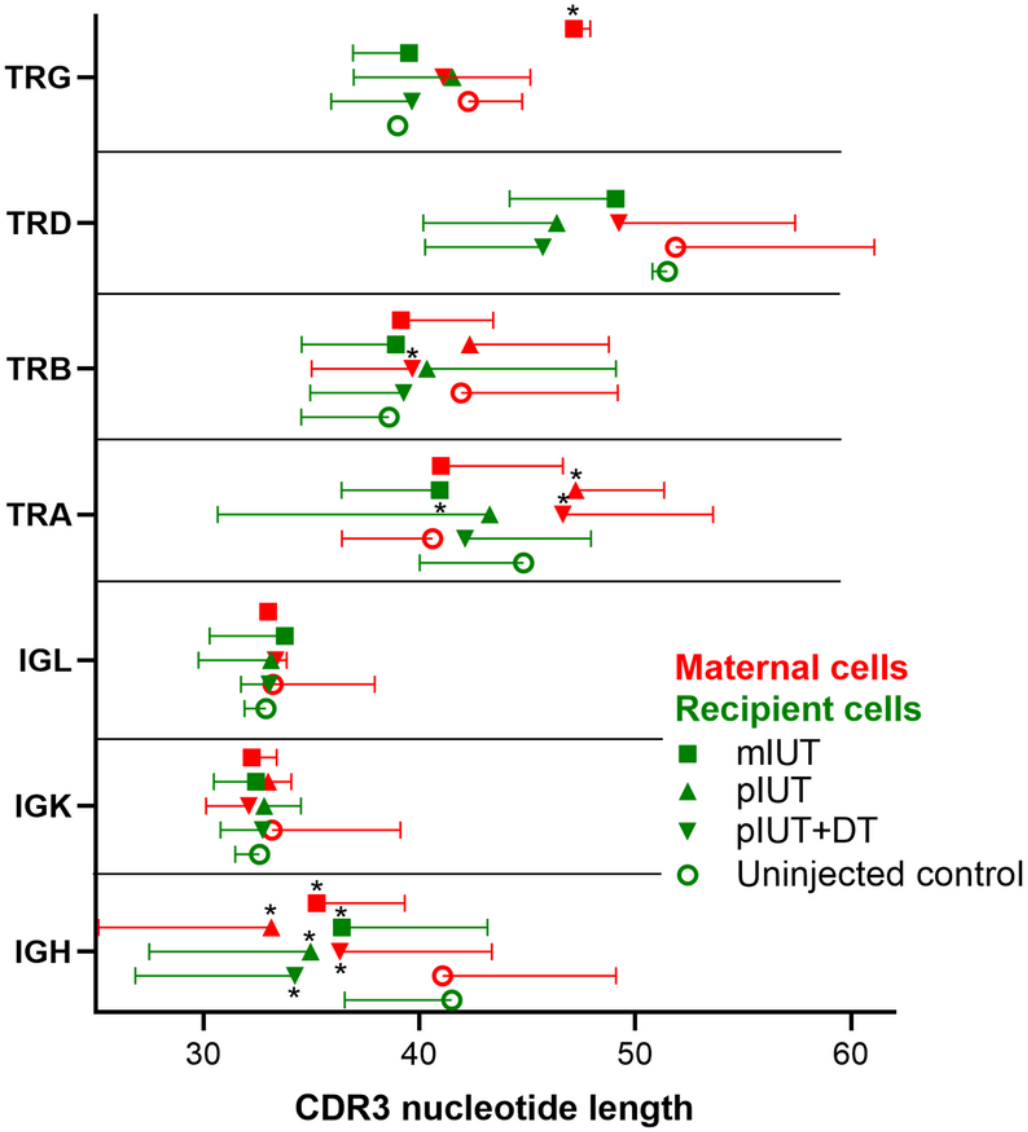


**Figure 6: TCR and BCR clonotypes show CDR3 nucleotide length changes in response to IUT.** Increased CDR3 nucleotide sequence lengths were observed in maternal TRG (from mIUT), TRA (pIUT, DT+ pIUT), and shorter lengths in maternal TRB (DT+pIUT), recipient TRA (mIUT), and maternal and recipient IGH (any IUT), compared to uninjected control pups. Mean ± SD are compared against controls, analysed with paired t-test.

## Figure 6

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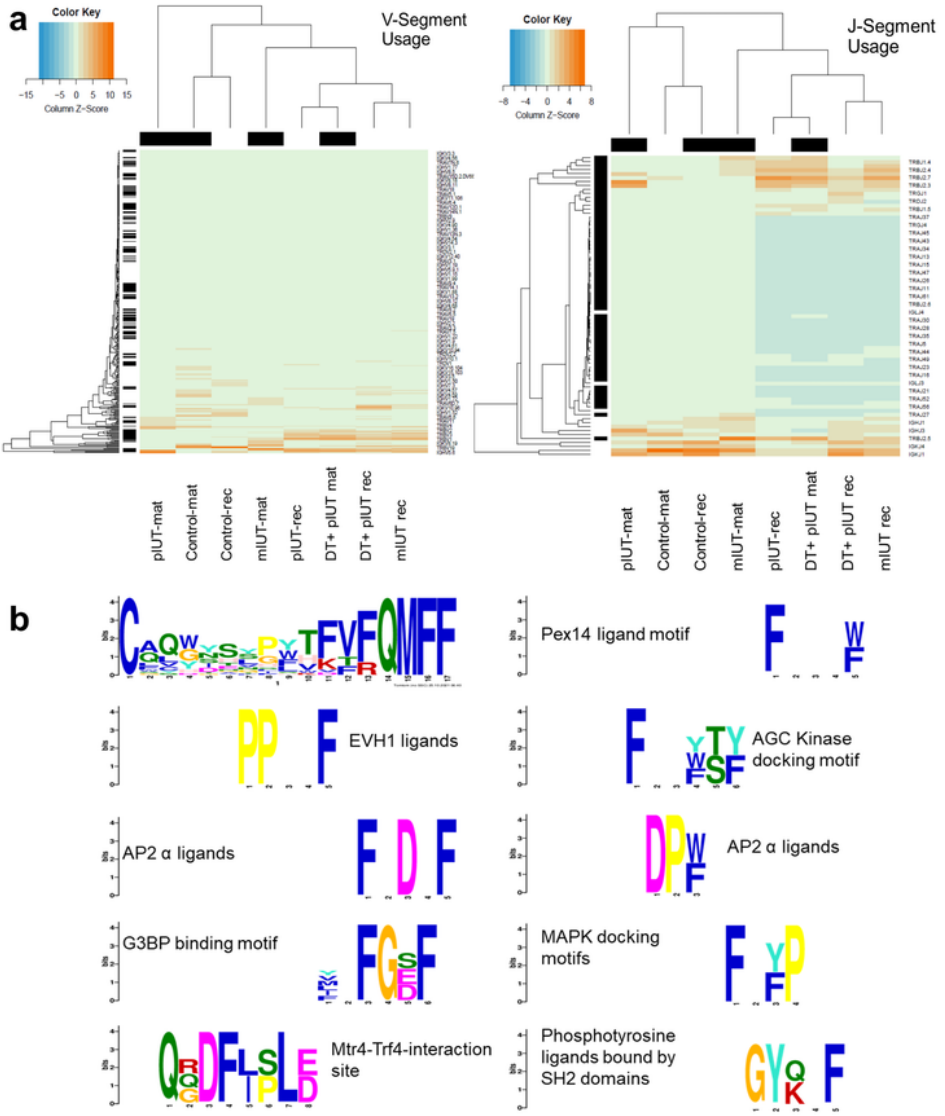


**Figure 7: CDR3 V-segment and J-segment analyses and functionality.** Similarities in variable V-segment and joining J-segment usage were observed between pIUT, DT+ pIUT and mIUT recipient-derived clonotypes and DT+ pIUT maternal-derived clonotypes, with upregulated IGH, IGK, TRA, TRB V- and J-segments, and TRG and TRD J-segments; TRA J-segments were downregulated (**a**). CDR3 amino acid sequences of the top 20 shared clonotypes demonstrated motif enrichment (listed in **b**) corresponding to Ras GTPase activating proteins, mitogen-activated protein kinases (MAPK) substrates, and signal transduction molecules, among others

### Figure 7

See image above for figure legend

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalDataCMLS.docx