Maternal Dendritic Cells Influence Fetal Allograft Response and Tolerance

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1 **Title page**

2 **Maternal Dendritic Cells Influence Fetal Allograft Response and Tolerance**

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10 **Author Contributions:**

KK, LGT, CNZM designed and performed research, analyzed data, and wrote the manuscript; NBJ, 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.

14 **Competing Interests:**

The authors have declared that no competing interest exists.

16 **Extended data and Supplementary Information** are available for this paper.

17

18 **Materials & Correspondence:**

19 Correspondence and requests for materials should be addressed to Dr. Citra NZ Mattar.

20

21 **Data Availability Statements:**

22 The datasets generated and/or analysed during the current study are available from the corresponding author
23 on reasonable request.

24 **Abstract**

25 Intrauterine hematopoietic cell transplantation (IUT), a promising therapy for congenital haematological disease,
26 is limited by subtherapeutic donor cell chimerism (DCC). Microchimerism of maternal immune cells (MMc)
27 trafficked into fetal IUT recipients may directly influence immune-mediated donor cell clearance. We investigated
28 if maternal dendritic cell (DC) suppression reduces recipient alloresponsiveness to donor cells, improving DCC.
29 IUT was performed at E14 in pregnancies resulting from crossing CD11c.DTR(B6) females and Balb/c males,
30 with semiallogenic Balb/c or C57BL/6, or fully allogenic C3H donor cells, 24h after administering diphtheria toxin
31 to the dam, transiently suppressing maternal DC. This resulted in reduced MMc in recipient fetuses, greatest
32 after Balb/c transplantation, also associated with the highest DCC, and lowest with C3H. Maternal-derived T-
33 cell receptor (TCR) clonotypes were enriched in IUT recipients and displayed substantially reduced diversity.
34 Recipient pups with reduced MMc increased expression of regulatory T-cell subtypes, reduced expression of
35 proinflammatory cytokines, and demonstrated enhanced TCR clonotype diversity. DCC was primarily related to
36 donor cell type and not influenced by MMc. Our data indicate that donor cell origin and MMc are distinct factors
37 determining IUT effectiveness. MMc independently influences DCC and recipient tolerance to donor cells and
38 may present novel therapeutic targets to improve transplantation outcomes.

39 **Summary**

40 *In utero* hematopoietic stem cell transplantation (IUT) represents potential cure for congenital hematological
41 disorders with advantages over conventional postnatal transplantation, particularly avoidance of myeloablation,¹
42 but immune barriers cause poor engraftment, hampering clinical application.²⁻⁵ Conventional dendritic cells
43 (cDC) activate CD8 and CD4 T-cells via subtypes cDC1 and cDC2 respectively,⁶⁻⁸ mediating antigen-specific
44 tolerance by altering helper T-cell (Th) balance^{9,10} and cytokine expression.¹¹⁻¹³ Active maternal-fetal trafficking
45 occurs throughout pregnancy,^{14,15} increasing after invasive procedures like IUT.¹⁶⁻¹⁸ Fetal alloresponsiveness to
46 IUT and subsequent donor cell chimerism (DCC) may be influenced by trafficked maternal cDC.^{19,20} To
47 understand how microchimeric maternal immune cells (MMc) in fetal IUT recipients affect DCC, we investigated
48 semi-allogenic and allogenic DCC following transient depletion of maternal cDC subtypes in a murine IUT model.
49 We observed lower MMc, expanded recipient regulatory T-cells, and subdued recipient alloresponsiveness to
50 donor cells. DCC, unaffected by reduced MMc, was highest with semi-allogenic (paternal) IUT at >2% for 12
51 weeks, and retained hematopoietic phenotype. T-cell (TCR) and B-cell receptor (BCR) repertoire diversity was
52 contracted within recovered MMc and expanded in recipient cells with semi-allogenic IUT; clonotype diversity
53 was variably influenced by maternal cDC depletion. Our results demonstrate that DCC depends on donor cell
54 origin, not MMc, and maternal and recipient alloresponsiveness can be manipulated via maternal cDC depletion.
55 Unique maternal and recipient TCR/BCR repertoires may serve as therapeutic targets to improve transplantation
56 outcomes. This clinically relevant data, similar to our previous study,¹⁶ supports paternal IUT for clinical trials
57 and encourages short-term maternal immune suppression at IUT.

58 Results

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

60 Diphtheria toxin (DT) administration to non-pregnant CD11c-DTR females depleted CD11c+MHC-II+cDC in
61 spleen, bone marrow (BM), peripheral blood (PB) and uterus (**Extended data Figure S1a**).²¹ Baseline cDC
62 (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
63 (**Figure 1a**). cDC1 was <0.1% at all timepoints, while cDC2 was returned to 0.86% in BM by day 7. Other
64 immune cells remained unaffected. Our IUT model (CD11c-DTR females crossed with BALB/c males) allowed
65 us to track donor, maternal and recipient cells separately. Intrahepatic IUT utilising maternal (B6, mIUT), paternal
66 (Balb/c, pIUT) or allogenic C3H (aIUT) cells produced survival rates in DT- and DT+ pups respectively of 92.0%
67 and 76.9% (uninjected controls, n=61), 75.0% and 79.6% (pIUT, n=60), 81.1% and 45.0% (mIUT, n=31), 100.0%
68 and 46.7% (aIUT, n=22, **Figure 1b**).

69 Next, we determined that microchimeric maternal cells (MMc) following DT+pIUT and DT-pIUT were similar in
70 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
71 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%),
72 and in DT+aIUT and DT-aIUT PB (0.04-0.08%); cDC1 and cDC2 were detected only in DT-mIUT and DT-aIUT
73 BM (0.03-0.04%, **Figure 1d**). Compared to DT-, we observed non-significant reductions in CD3, CD4, CD8,
74 CD19 in DT+pIUT and DT+mIUT BM, and higher CD3, CD4, CD8 in DT+aIUT BM (**Figure 1d**), with non-
75 significant increases in CD3, CD4, CD8 in DT+pIUT and DT+mIUT PB, and reductions in all cells in DT+aIUT,
76 particularly NK1.1 ($10.4\pm 10.4\%$ v $29.9\pm 10.7\%$, $p<0.0001$). Thus, maternal DC depletion influences MMc
77 differently depending on IUT.

78

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

80 We observed higher donor cell chimerism (DCC) in BM (DCC^{BM}) in DT-pIUT than in DT-mIUT and DT-aIUT
81 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-
82 mIUT showed higher DCC^{PB} than DT-aIUT and DT+mIUT (0w, $p<0.05$). With DT+pIUT, DCC^{BM} was maintained
83 >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
84 DT-mIUT and DT+mIUT remained <1% while DCC^{PB} declined rapidly from $11.07\pm 0.07\%$ (0w, DT-mIUT), and
85 remained <1% (0-12w, DT+mIUT). DCC^{BM} and DCC^{PB} in DT-aIUT and DT+aIUT remained <1% (0-12w).
86 Recovered DCC^{BM} and DCC^{PB} showed similar proportions of Lin-Sca1+c-Kit+ (LSK), CD48+ and CD150+
87 indicating the presence of long-term repopulating HSC (**Extended data Figure S1b**). We deduced that post-
88 IUT maternal cell trafficking was an active process, as we observed significant reductions in CD3 and CD19 in
89 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 ($p<0.0001$, **Extended 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+plUT, 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+plUT compared to DT-plUT (where it is the most prevalent cell) at 0-12w ($p<0.005$) and was also higher than cDC subsets in DT+plUT (4w, $p<0.05$); no other differences were observed in MMc between groups (**Figure 2c, d**). DT+plUT and DT-plUT 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.

When examined for tolerance, re-exposure of DT+plUT splenocytes to Balb/c cells elicited greater expression of CD4 effector memory (T_m), regulatory (Treg) and CD25+Treg compared to DT-plUT and untreated controls ($p<0.001$, **Figure 2g**), and re-exposure to B6 cells produced higher CD4 Treg compared to DT-plUT and controls ($p<0.001$). DT+plUT recipients showed greater fold-change in CD8 central T_m, effector (T_{eff}), Treg and CD62L+CD25+Treg ($p<0.001$) when challenged with B6 cells, compared with controls and DT- recipients (**Figure 2h**). DT-plUT produced greater fold-changes in cytokines associated with helper T-cells,²² when stimulated with Balb/c or B6 cells, while a relatively lower response was observed for DT+plUT (**Figure 2i-n**). Postnatal boost of maternal cells increased CD3 and decreased CD19 levels only in lymph nodes in DT+plUT (**Extended data Figure S2b-f**), but no donor-specific antibodies were observed following IUT or postnatal boost (**Extended data Figure S2g**).

Though DCC^{BM} and DCC^{PB} were $<0.2\%$ in DT+mIUT, we were able to analyse donor immune cell components. DCC^{BM} in DT+mIUT contained higher CD3, CD8, CD19 than cDC, NK1.1 ($p<0.05$), and compared to DT-mIUT, DCC^{BM} contained higher CD3, while DCC^{PB} showed lower CD3, CD8 ($p<0.05$, **Figure 3a,b**). 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 T_m, CD4 and CD8 T_{eff}, and CD4 and CD8 CD25+Treg, CD62L+CD25+Treg when stimulated with Balb/c cells only (**Figure 3g, h**); only low-level responses to B6 cells were elicited. Cytokine expression was much lower in mIUT than plUT recipients (<6 -fold increase over untreated controls). DT+mIUT produced a higher expression of largely inhibitory cytokines (IL5, IL6, IL10, FOXP3, TGF β 2) in combinations associated with Th2, Th1/Th2, Th17 cells, following B6 and Balb/c stimulation (**Figure 3i-n**).

122 In contrast, DT+alUT and DT-alUT resulted in only microchimeric DCC^{BM} and DCC^{PB}, with similar donor immune
123 cell profiles. No differences in MMc, trafficked maternal immune cell profile or recipient immune cell profile were
124 observed (**Extended data Figure S3a-f**). Significant fold-change increases in CD4 central and effector T_m, and
125 CD4 and CD8 T_{eff}, CD25⁺Treg and CD62L⁺CD25⁺Treg were observed in DT+alUT (**Extended data Figure**
126 **S3g,h**), which also significantly increased expression of all cytokines except FOXP3 when stimulated with C3H,
127 compared to DT-alUT, implying proimmune helper T cell enhancement. alUT produced cytokine responses
128 close to control levels when stimulated with B6 and Balb/c, except for IL-17a, IL-17f, IL-22, augmented with
129 DT+alUT (**Extended data Figure S3i-n**).

130 We attempted to evaluate regulatory B-cells (Breg) which lack unique phenotypic markers.^{23,24} pIUT and mIUT
131 augmented IL10, IL5, IL6, TGF- β 2, FOXP3 expression (**Figure 2j,m**; **Figure 3j,m**) suggesting the presence of
132 activated Breg in response to IUT. There were no differences in CD19 post-transplantation (**Figure 2e,f**; **Figure**
133 **3e,f**). Thus, maternal DC depletion may promote a tolerogenic response through both Treg and Breg.

134

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

136 To further parse maternal and recipient immune interactions, we analysed gene expression profile and T-cell
137 (TCR) and B-cell (BCR) receptor repertoires in MNC isolated from pIUT and mIUT recipients. Top 3000 up- and
138 down-regulated genes in each group – uninjected (DT-, n=17), DT-mIUT (n=7), DT-pIUT (n=5), DT+pIUT (n=5)
139 – were identified by enrichment score (ES) >1.3. Within treatment groups, recipient and maternal cells shared
140 483-736 common genes (**Figure 4a**). With mIUT, 26/174 enriched recipient gene clusters and 25/171 maternal
141 clusters represented RNA and protein metabolism, hemopoiesis and immune system development, also
142 represented by 21/169 recipient clusters and 49/177 maternal clusters following pIUT. With DT+pIUT, 20/153
143 recipient clusters additionally represented T-cell regulation. Upregulated DT-pIUT and DT+pIUT Maternal
144 clusters represented mitogen-activated protein kinases (MAPK) cascades, T-cell activation, and immune system
145 development. All groups shared 48 common genes, of which 6/40 highly enriched clusters represented cytokine
146 stimulus response, immune system regulation, B-cell mediated immunity and adaptive immune response
147 (**Figure 4b**).

148 Compared to uninjected controls, retrieval frequency of maternal-derived TCR and BCR clonotypes increased
149 from 0.02% to 2.04-3.13% following mIUT and pIUT respectively (**Table 1**), and these showed reduced diversity,
150 with Hill numbers (order 1, exponential of Shannon-Wiener indices)²⁵ of 18-29, compared to 941 in controls.
151 DT+pIUT decreased maternal clonotype retrieval by 17.3-fold and increased diversity towards baseline (Hill
152 number of 447). In contrast, mIUT and pIUT recipient-derived clonotypes (retrieval frequency 0.02-0.15%)

153 showed increased diversity (Hill numbers 528-3728 v 64 in controls). DT+plUT reduced diversity in recipients
154 (Hill numbers from 3728 to 1637).

155 Maternal- and recipient-derived TCR and BCR clonotypes were similar in uninjected pups; BCR immunoglobulin
156 heavy (IGH), κ (IGK), λ (IGL) chains made up ~81% of maternal and 75% of recipient clonotypes (**Figure 4c**).
157 Expansion of TCR β -chain (TRB) clonotypes and reduction in IGH were observed among maternal-derived
158 clonotypes following mIUT and DT-plUT. DT+plUT produced further increases in TRB, TRA (α -chain), TRD (δ -
159 chain), IGH, IGK relative to DT-plUT. Among recipient-derived clonotypes, we observed expanded TRB, TRG
160 (γ -chain), and reduced IGH, IGK following mIUT and plUT, and DT+plUT further reduced TRB and increased
161 IGK and IGH.

162 Mostly higher-order (3+) clonotypes were encountered with IUT (**Figure 4d,e**). Top 20% of clonotypes (Quantile
163 1, Q1) were most abundant in uninjected controls, and clonotypes in Q2 to Q5 were more abundant in IUT
164 recipients. Individual abundances of top 5 maternal-derived clonotypes in each group were expanded with IUT
165 and diminished with DT+ (**Figure 4d**), while recipient-derived top 5 clonotypes were most abundant in uninjected
166 pups (**Figure 4e**). We observed a large number of public maternal-derived clonotypes between DT+plUT and
167 controls (**Figure 4f**), and a substantial number of public recipient-derived clonotypes between mIUT, plUT and
168 DT+plUT (**Figure 4g**).

169 Complementarity-determining region 3 (CDR3) incorporates the VDJ recombination junctions, accounting for
170 most of the repertoire variation mediating specific antigen recognition.²⁶ CDR3 regions of naïve and antigen-
171 experienced clonotypes are longer and shorter respectively, reflecting antigen-driven selection.²⁷ We found a
172 significant shift towards longer CDR3 in maternal TRG (from mIUT) and TRA (DT-plUT, DT+plUT), and shorter
173 CDR3 in maternal TRB (DT+plUT), recipient TRA (mIUT), and maternal and recipient IGH (all groups). Analyses
174 of gene segment usage to functionally characterize these clonotypes by hierarchical clustering indicate
175 similarities in V/J-segment usage between plUT, DT+plUT and mIUT recipient-derived clonotypes and DT+plUT
176 maternal-derived clonotypes, where IGH, IGK, TRA, TRB V/J-segments, and TRG and TRD J-segments, were
177 upregulated and TRA J-segments were downregulated. We also observed uniqueness in V/J-segment usage
178 between uninjected control, mIUT and plUT clonotypes (**Extended data Figure S5a**). Functionality of the top
179 20 shared clonotypes, assessed by comparing CDR3 amino acid sequences to protein databases using
180 Tomtom,²⁸ revealed motif enrichment corresponding to production of Ras GTPase activating proteins, MAPK
181 substrates, and signal transduction molecules, among others (**Extended data Figure S5b**). These data show
182 that both maternal and recipient immune cells respond to donor cells and that only maternal TCR and BCR
183 diversity is affected by maternal DC depletion.

184 Discussion

185 To our knowledge, this is the first report of maternal immune cells “primed” on exposure to donor cells
186 influencing recipient immune responses to IUT. We demonstrated that DT+piUT reduced trafficking of
187 maternal cDC, expanded regulatory T-cells and restricted pro-immune cytokines, contrasting with largely
188 alloreactive responses to mIUT and aIUT. Inducing the appropriate balance of Th1/Th17/Th2, which influence
189 allograft tolerance or rejection,^{22,29} may be critical to improving IUT transplantation tolerance. Maternal
190 TCR/BCR clonotypes displayed substantially reduced diversity post-IUT, restored with DC depletion, while
191 recipient-derived TCR enrichment was unaffected. Our findings indicate that DCC and MMc are separate yet
192 equally important determinants of IUT effectiveness. piUT produced the highest and most persistent DCC. In
193 contrast to other reported models using maternal donor cells,^{30,31} the model for IUT clinical trial NCT02986698,
194 we observed microchimerism post-mIUT despite reduced MMc, similar to our earlier work.¹⁶ Thus, DCC
195 appears primarily dependent on cell origin, not MMc. We demonstrated that maternal cell trafficking is an
196 active process, the quality of which appears to influence the fetal recipient’s immune response to donor cells.
197 Taken together, piUT resulted in both highest DCC and lowest MMc, and maternal DC depletion further
198 dampened recipient alloresponsiveness through upregulated Treg, the possible presence of Breg, and
199 downregulated proimmune Th cells. aIUT produced both poorest DCC and most robust alloresponsiveness,
200 contrasting with data from other IUT models in which allogenic donor cells have a competitive advantage.³²⁻³⁶
201 Although reduced MMc was associated with increased Treg and Breg cytokine expression, upregulated Teff
202 and an overwhelmingly proimmune cytokine response were present, expediting allogeneic cell rejection.
203 Maternal DC may also present donor antigen to recipient immune cells, influencing the quality of immune
204 response. Enhanced Treg production following IUT, particularly of CD62L+CD25+FOXP3+ Tregs, protects
205 against graft-v-host disease,^{37,38} and together with putative Breg (expressing IL10, IL5, IL6, FOXP3, TGF-β),
206 probably influenced the resulting tolerogenic or immunogenic responses.^{23,24} Human transplantation data
207 indicate that persistent recipient cells inhibit donor haematopoiesis and TCR reconstitution.³⁹⁻⁴¹ IUT adds an
208 interesting dimension to this, wherein the maternal immune system is the other “recipient” interacting with
209 donor cells, and both maternal and recipient immune systems may impact long-term engraftment and
210 maintenance of tolerance.

211 Trafficked maternal TCR and BCR clonotypes showing reduced diversity, variable CDR3 lengths and V- and J-
212 segment usage indicate dynamic acquisition of allo-specificity, further underscoring the “sensitization” of
213 maternal cells to transplanted donor cells. Donor cells transplanted directly into the fetus may leak into
214 maternal circulation and participate in maternal immune modelling, selecting and expanding particular
215 clonotypes for trafficking, similar to native fetal microchimerism.⁴² The return of DT+ maternal clonotypes to
216 higher baseline diversity supports this postulation. Increased recipient TCR diversity confirm that treated
217 fetuses mount antigenic responses even to haploidentical cells. Clinically-poor responders to postnatal HSCT
218 show lower TCR diversity,^{26,43,44} thus recipient TCR repertoire may be a useful biomarker of transplant
219 rejection.⁴⁵

220

221 Our murine model permitted specific depletion of antigen-presenting cDC in pregnant mice, for which there
222 are no published data. cDC depletion at E13 would have endured for the remainder of the pregnancy
223 (parturition ~E20), resulting in naïve maternal cDC trafficked to fetal recipients. Of particular interest is
224 depletion of uterine DC, a unique subtype critical to pregnancy-related tolerance and rejection,⁴⁶ capable of
225 eliminating fetal cells, which concentrate in maternal circulation towards parturition,⁴² while not rejecting the
226 fetus.^{47,48} Allogenic fetal cells presented by uterine DC can prime maternal tolerance towards paternal antigens
227 via expansion or elimination of alloreactive or regulatory lymphocytes.⁴⁹⁻⁵¹ Donor cells transplanted in mid-
228 gestation likely leak into maternal circulation, prompting reciprocal trafficking of primed maternal cells.
229 Indeed, with DC depletion, maternal TCR clonotypes showed markedly greater diversity, and piUT and miUT
230 recipients expanded Treg subsets and upregulated less immunogenic cytokines. Further investigation into
231 uterine DC may intriguingly reveal a unique target for in-vivo or ex-vivo modulation to enhance transplantation
232 tolerance.¹²

233

234 Our data raise the exciting possibility of utilising unique maternal and recipient TCR/BCR repertoires in multiple
235 fashion for IUT, and by extension other cellular fetal therapies, by serving as biomarkers of engraftment and
236 immune tolerance, as therapeutic targets to improve transplantation tolerance, e.g., by transducing regulatory
237 sequences into high avidity clonotypes, and as prognosticators of transplantation outcomes. Robust graft

238 tolerance required to maintain life-long engraftment carries several layers,⁵² that, in combination, may even
239 permit repeat transplantation, including limiting maturation of high-avidity alloreactive T-cells and expanding
240 high-avidity Treg.⁵³⁻⁵⁵ Selective expansion of regulatory TCR clonotypes with distinctly tolerogenic phenotypes
241 may be an individualizable strategy through which to achieve such tolerance.⁵⁶ Bregs maintain immune
242 tolerance, but are more difficult to identify due to the absence of unique markers.^{57,58} Though maternal and
243 recipient TCR clonotypes underwent the greatest expansion, we observed an increase in recipient-derived BCR
244 clonotypes along with upregulated Breg-associated cytokines. This suggests that putative regulatory BCR
245 clonotypes are represented and may be valuable components of IUT tolerance. Our data may influence clinical
246 management even in the short-term, supporting the transplantation of paternal donor cells in clinical trials or
247 short-term maternal immune-suppression at the time of IUT, similar to immunotherapy for recurrent
248 miscarriages.⁵⁹ Our work is limited by transplantation of a small donor inoculum and limited monitoring for 12
249 weeks, while pandemic restrictions curtailed our assessment of DT+mIUT and aIUT immune repertoires.
250 Additionally, in the limited number of mIUT and aIUT recipients harvested, donor chimerism was very low
251 which may affect accuracy of donor cell analyses. Transplantation of larger doses to achieve therapeutic DCC
252 and a longer surveillance of maternal/recipient immune profiles in larger animals will be informative for future
253 clinical translation.

254 **Methods**

255 **Reagents and antibodies.** Roswell Park Memorial Institute Medium (RPMI 1640), fetal bovine serum (FBS),
256 non-essential amino acids, GlutaMAX, 2-Mercaptoethanol, penicillin-streptomycin antibiotics and collagenase
257 type IV powder were obtained from Gibco (ThermoFisher Scientific, Waltham, MA). Mitomycin C was obtained
258 from ALPS Pte Ltd (National University Hospital Pharmacy, Singapore). Diphtheria Toxin (DT) and bovine serum
259 albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO). Ficoll-Paque PLUS were obtained from GE
260 Healthcare (Chicago, IL). RBC lysis buffer was obtained from Biolegend (San Diego, CA). Cytofix/Cytoperm
261 solution, Perm/Wash buffer, staining buffer, brilliant stain buffer and fixable viability stain 620 were obtained from
262 BD Biosciences (San Jose, CA). AutoMACS rinse solution was obtained from Miltenyi Biotec (Singapore).
263 FastStart Universal SYBR green master mix (Roche Life Science, Germany) were obtained from Sigma-Aldrich.
264 DNA oligos for qPCR were obtained from Integrated DNA Technologies (IDT, Singapore). VersaComp antibody
265 capture compensation beads were obtained from Beckman Coulter (Singapore). Fluorescent-activated cell
266 sorting (FACS) antibodies were obtained from BD Biosciences, Biolegend and Life Technologies (ThermoFisher
267 Scientific). A list of abbreviations used in the manuscript can be found in extended data table (S1).

268 **Animal experiments.** Inbred strains CD45.2 BALB/c (H-2K^d) and C3H/HeNTac (H-2K^k referred to as C3H) were
269 obtained from In Vivos (Singapore). CD45.1 C57BL/6 mice and CD11c-DTR female mice (B6.FVB-
270 1700016L21RikTg^{(Itgax-DTR/EGFP)⁵⁷Lan/J}) were purchased from The Jackson Laboratory (Bar Harbor, ME) and
271 maintained in a specific pathogen-free facility at NUS. BALB/c males and CD11c-DTR females were time-mated
272 for IUT at E14. When performing animal experiments, pregnant mice were randomly chosen for IUT and for
273 downstream experiments, as the probability of getting pregnant dams in a group of breeding mice occur by
274 chance. All mouse experiments were performed according to IACUC (Institutional Animal Care and Use
275 Committee) approved protocols (BR16-1203 and R16-1200) at the National University of Singapore (NUS),
276 Singapore.

277 **Transient DC suppression.** CD11c-DTR females were given intraperitoneal injection of DT (5ng/g of body
278 weight) and controls were given saline. The females were harvested on day 0, 1,2,4 and 7 days post injection,
279 BM, PB, spleen and uterine horn organs were analysed for CD3, CD4, CD8, CD19, NK1.1, CD11c, MHC-II,
280 XCR1 and CD172a immune parameters by flow cytometry. Conventional DC (cDC) were identified by the
281 double positive population of MHC-II and CD11c. From cDC, cDC1(XCR1+) and cDC2 (CD172a+) cells were
282 identified.

283 **Intrauterine transplantation (IUT) and postnatal boost.** Fresh donor bone marrow mononuclear cells (BM-
284 MNC) were prepared as previously described.¹⁶ 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
285 on E14 and all fetuses received intrahepatic injection of 5E+6 donor inoculum, as described previously.¹⁶
286 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
287 IUT (aIUT). Uninjected controls were offspring of DT+ or DT- dams which did not undergo intrauterine
288 transplantation. After littering, pups were nursed by their mothers and weaned at 4 weeks. Fetal cells were
289 harvested from F1 cross-bred hybrid pups sacrificed between postnatal weeks 1 to 12 to assess donor cell
290 chimerism (DCC), trafficked maternal cell microchimerism (MMc) and fetal immunological responses in
291 peripheral blood (PB) and BM. In selected offspring in each group, postnatal boost was performed with 5E+6
292 donor cells after 12 postnatal weeks administered via retro-orbital route, without bone marrow ablation.

295 **Isolation of MNC and FACS analysis.** MNC were isolated from various organs and approximately 1E+6
296 cells/tube were stained for surface markers to differentiate trafficked maternal cells (H2K-b+), and fetal recipient
297 cells (double positive H2K-b+, H2K-d+) as reported.¹⁶ Donor cells were identified from fetal recipient cells by the
298 expression of CD45.1+ (maternal donor cells), H2K-d+ (paternal donor cells), H2K-k+ (C3H donor cells) surface
299 markers. Further, cells were stained for T cells (expressing CD3, CD4, CD8, NK1.1), B cells (CD19), dendritic
300 cells (CD11c, MHC-II, XCR1, CD172a), HSC markers (Sca1, c-Kit, CD48, CD150), hematopoietic lineage
301 markers (CD11b, GR1, TER119, CD3, CD19, B220). All antibody-stained cells were analyzed using BD X-20
302 Fortessa™ flow cytometer (BD Biosciences, Franklin Lakes NJ, accessed at the NUS Life Science Institute),
303 The list of antibodies used for flow cytometry were provided in extended data table (S2). Each antibody was
304 validated with positive control samples as per manufacturer's instructions. All raw data files were compensated
305 and analysed using FlowJo™ software (FlowJo LLC, Ashland OR). Percentage of donor cells and maternal
306 trafficked cells were calculated from the total live MNC. Percentage of immune populations were calculated from
307 the respective total population of MMc, donor cells and recipient cells and the percentage of HSC markers were
308 calculated from the total donor cells.

309 **Mixed lymphocyte reactivity assay (MLR) for functional T cell activity.** Splenocytes (responder cells) were
310 isolated from sacrificed F1 cross-bred IUT and naive (from uninterrupted pregnancies) pups. Lymphoid tissue
311 MNC from BALB/c, B6 or C3H mice, treated with mitomycin C (50µg/ml), served as stimulator cells. MLR was
312 performed as reported.¹⁶ Cells were harvested after 72h post culture and analysed for T cell subset surface
313 markers (CD4, CD8, CD25, CD62L, CD44) and intracellular marker FOXP3 in the responder cells by flow

314 cytometry; part of the cells was used for RNA extraction and cytokine gene expression analysis by RT-qPCR.
315 Markers for phenotypes: central memory T cells (central Tm, CD44+CD62L+), effector memory T cells (effector
316 Tm, CD44+), effector T cells (Teff, CD25+), regulatory T cells (Treg, FOXP3+).

317 **Quantitative gene expression analysis.** Total RNA extraction and qPCR was performed as described
318 previously.¹⁶ List of qPCR primers can be found in the extended data table S3. Gene expression values were
319 normalized with endogenous control GAPDH. Relative quantification of each target gene studied was calculated
320 by the $\Delta\Delta CT$ method.

321 **Bulk RNA Sequencing.** Trafficked maternal and recipient cells were isolated from 1-week-old F1 hybrid
322 neonates (groups consists of uninjected offspring (no IUT, DT-, n=17), mIUT (DT-, n=7), pIUT (DT-, n=5) and
323 DT+pIUT (n=5)) by magnetic cell sorting (MACS), followed by FACS, using H2K-b and H2-Kd antibodies
324 respectively. Isolated maternal trafficked cells from each neonate were not sufficient for RNA-seq analysis as
325 individual samples. We pooled the samples from a group of neonates to generate sufficient number of cells for
326 sorting and performed RNA-seq. Thus, we do not have replicates for RNAseq samples that were analysed, but
327 each sample represents a certain number of harvested animals. We were unable to isolate sufficient samples
328 from DT+ mIUT and aIUT groups for analyses. Total RNA was isolated from the sorted cells using RNeasy micro
329 kits (Qiagen) with on-column RNase-free DNase digestion and eluted carrier-free. Total RNA was quantified,
330 and the quality assessed using Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA), before
331 cDNA library preparation. Samples were sequenced on a flow cell using HiSeq 4000 system (Illumina, San
332 Diego, CA), with a read length of 150bp and 100 million reads per sample. The raw reads were analyzed with
333 the RNAseq pipeline from nf-core⁶⁰ using the reference genome (GRCm39) and gene annotation (M26) from
334 GENCODE (<https://www.gencodegenes.org/>). The gene read count table generated by featureCount in the
335 RNAseq pipeline was analysed using edgeR software.^{61,62} The dispersion value was set at 0.4 as recommended
336 in edgeR for experimental setups with no replicates.

337 **Gene expression.** Using log of fold-change (logFC) data, we selected the most up- and down-regulated genes
338 (1500 each, for a total of 3000) from each treatment group to organize using an online Venn diagram generator
339 (<http://bioinformatics.psb.ugent.be/webtools/Venn/>) to single out common genes across all the groups. We
340 analysed functions of enriched genes using the Database for Annotation, Visualisation and Integrated Discovery
341 (DAVID v6.8, <https://david.ncifcrf.gov/>).^{63,64}

342 **TCR and BCR repertoire analyses.** Raw reads were analyzed by MIXCR performed with settings of analysis
343 of random fragments, RNA starting material and using the provided Mus musculus dataset, all others remaining

344 as default settings.^{65,66} Results from MIXCR were imported into VDJtools (<https://github.com/mikessh/vdjtools>)
345 to plot the clonotypes in PlotQuantileStats.⁶⁷

346 **Detection of donor specific antibodies (DSA).** Serum collected from harvested pups and serum collected
347 after donor booster injections were used for DSA. For generating positive control sera, wild-type B6 mice were
348 sensitized with maternal (B6/CD45.1), paternal (BALB/c) or allogenic (C3H) splenocytes at a dose of 2E+7 cells,
349 injected intra-peritoneally at an interval of 2 weeks. Sera was collected after 14 days. DSA assay was performed
350 using respective splenocytes, as described previously.¹⁶

351 **Statistical analyses.** Continuous data were analysed using Analysis of variance (2way ANOVA) with Tukey's
352 multiple comparisons test, with a single pooled variance and multiple t tests for comparisons of individual
353 parameters. Statistical significance was determined at $\alpha=5.0\%$. Values are expressed as mean $\pm$ standard error
354 of the mean (SEM). Pearson correlation coefficient was also used assuming linear relationships between the
355 variables tested. Analyses were performed with GraphPad Prism version 9 for Windows (GraphPad Software,
356 San Diego, CA, www.graphpad.com). Error bars were not provided on figure 2a-f, figure 3a-f and extended
357 figure S3a-f, as they may mask a clear view of the graph bars. Instead, respective raw data (mean $\pm$ SEM), were
358 provided in the supplementary table 1-3). All experimental data were collected and analysis were performed by
359 separate team of staffs. Investigators were blinded for the current study. The datasets generated and/or
360 analysed during the current study are available from the corresponding author on reasonable request.

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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) <small>*exponential of the Shannon-Weiner Index</small>
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 (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 (maternal DC suppression)	18579	3064	3.26E-4	1637

531

532 **Fig. 1: Transient suppression of maternal dendritic cells with diphtheria toxin resulted in reduced**
 533 **maternal immune cell trafficking to fetal recipients following intrauterine transplantation of semi-**
 534 **allogenic and fully allogenic donor cells, and effect on donor cell chimerism**

535 Selective reduction of conventional dendritic cell (cDC) and subtypes (cDC1, cDC2) in various organs of non-
 536 pregnant CD11c.DTR females (n=5) after administering diphtheria toxin (DT, arrows, **a**). Intrauterine
 537 transplantations of maternal (n=31, mlUT) paternal (n=60, plUT) and fully allogenic (n=22, alUT) cells
 538 performed at E14 (**b**), with (DT+) or without DT (DT-). Maternal immune cell microchimerism (MMc) was similar
 539 in DT- and DT+ with each IUT (**c**), higher in alUT v plUT, and higher in DT+ bone marrow (BM) v peripheral
 540 blood (PB) after mlUT (black*). Donor cell chimerism (DCC) was highest with DT- plUT in BM and PB (black*),
 541 and higher in mlUT PB v BM (red*). With DT+, DCC was maintained in plUT BM (black*), and was higher in
 542 mlUT BM v PB (red*). Maternal cDC, CD3, CD4, CD19 cells were non-significantly reduced in DT+ recipient BM
 543 and higher in PB (**d**). cDC and subtypes were only found in DT- and DT+ alUT recipients. Data represent
 544 mean±SEM, analysed by two-way ANOVA with Tukey's multiple comparisons test.

Fig. 2: Immune profiles of engrafted donor, trafficked maternal and recipient cells, T cell profile and cytokine 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 maternal microchimerism with lower levels of all immune cells, and lower cDC, CD19, NK1.1 relative to CD3 (p<0.05, **c,d**), in bone marrow (BM) and peripheral blood (PB) respectively. 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, black*). Data represent mean±SEM, analysed by two-way ANOVA with Tukey's multiple comparisons test.

Fig 3: Immune profiles of engrafted donor, trafficked maternal and recipient cells, T cell profile and cytokine expression following maternal cell transplantation

Donor cell chimerism was low in DT+ (n=8) and DT- (n=7). In DT+, CD3 was higher in BM and 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 $\pm$ SEM, analysed by two-way ANOVA with Tukey's multiple comparisons test.

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Fig. 4: 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).

Supplementary Files

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- [SupplementaryInformation181221.pdf](#)
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