Chapter 5: Investigation of host lymphoid progenitor cell (LPC) recruitment in iTEC-based RTOC

5.1. Introduction and aims

Chapter 4 investigated the role of fetal thymic mesenchyme (FTM) in the iTEC-RTOC system and identified it could be partially compensated for by increasing iTEC numbers and number of RTOC transplanted per recipient. The aforementioned 'iTEC-MultiRTOC' system will be taken forward in this Chapter. Lymphoid Progenitor Cell (LPC) recruitment is a vital function of native TEC and therefore the ability of iTEC to carry out this function is essential in understanding their ability to support long-term T cell reconstitution in the host. In addition, the requirement of donor DN thymocytes, which has not previously been examined, limit the translational relevance of the system, and introduces further variation.

In Chapter 5 the ability of iTEC-MultiRTOC to recruit host LPCs and support their commitment to the T cell lineage and develop into functional T cells alongside the requirement of DN thymocytes in the system will be investigated. Together, Chapter 4 and 5 will allow for the identification of the minimum cellular requirements for iTEC-RTOC transplantation. The data presented provide important information relevant to the development of a transplantable synthetic thymic organ that could be used for clinical purposes.

5.2. Can iTEC-RTOC recruit host lymphoid progenitor cells?

An additional component of generating a synthetic thymus for transplantation is provision of thymocytes prior to or after transplantation, in order to repopulate the lymphopenic host environment with a fully functional T cell repertoire. An important function of TEC is the ability to recruit circulating host bone marrow-derived lymphohaematopoietic progenitor cells (LPCs). Thymus homing of LPCs is important for long-term T cell reconstitution of the host and continuous seeding of the thymus ensures that a diverse T cell repertoire is generated and maintained. LPC thymic colonization occurs due to interaction of platelet (P)- selectin on thymic endothelial cells and platelet-selectin glycoprotein ligand-1 (PSGL-1) on LPCs, and interaction of chemoattractant CCL25 produced by TEC with LPC receptor CCR9 (Wurbel *et al*., 2000; Rossi *et al*., 2005; Scimone *et al*., 2006; Schwarz *et al*., 2007).

Although provision of an initial dose of immature donor thymocytes takes place prior to transplantation it is not known whether grafted iTEC have the ability to recruit host LPCs. If iTEC survive long-term *in vivo* and simultaneously have the ability to recruit host lymphoid progenitors, this could offer a solution for treating T cell immunodeficiencies in patients, including those associated with ageing. Additionally, removal of donor thymocytes from the iTEC RTOC system could reduce the risk of graft vs host disease (GVHD) and result in a more clinically relevant system.

5.2.A. Experimental design: tracing host and donor T cells in recovered iTEC grafts

CD45, expressed on the surface of all haematopoietic cells, has two distinct alleles in the mouse that can be identified with antibody staining. These alleles are CD45.1 (Ly5.1) and CD45.2 (Ly5.2). WT C57BL/6J mice carry the CD45.2 allele and Ly5.1 homozygous mice, bred in house, are C57BL/6J mice that are homozygous for the CD45.1 allele.

Therefore, to test whether iTEC-RTOC can attract host LPCs, an experiment was set up in which all cells within the RTOC donor, including DN thymocytes, were obtained from CD45.2^{+} WT C57BL/6J x C57BL/6J embryos. These were transplanted under the kidney capsule of Ly5.1 (CD45.1) homozygous mice, using the MultiRTOC protocol. Development of both donor (CD45.2^{+}) and host (CD45.1^{+}) thymocytes present in recovered grafts was then investigated at two-, four- and six-weeks post transplantation (Figure 5.1.A).

As discussed previously in Chapter 4 iTEC-MultiRTOC performed better that single iTEC-RTOC in supporting T cell development into the DP stage but did not perform as well as iTEC+FTM-RTOC. During this experiment iTEC-MultiRTOC and iTEC+FTM-MultiRTOC (each RTOC contained 50,000 FTM cells) were both tested, to ensure FTM did not have an impact on LPC recruitment and that it did not have an additive effect with the increase in iTEC numbers (Figure 5.1.B). Negative control Cre MEFs were not included, to reduce number of mice used for experiments as previous experiments show that Cre MEFs do not support any T cell development upon transplantation.

The flow cytometry panel used to investigate T cell differentiation in the recovered grafts included 7-AAD, CD45.1, CD45.2, CD3ε, TCRβ, CD4, CD8, CD25, CD44, CD69 and CD62L. DN T cells can be further divided into DN1, DN2, DN3 and DN4

based on the expression of CD44 and CD25 (Godfrey *et al*., 1993). SP T cells have been shown to progress through intermediate stages of maturation before reaching a fully mature state ready for travel to the periphery of the animal. CD69 is a marker of TCR-mediated positive selection and can be used to identify semi-mature SP that are undergoing or have recently completed positive selection (Yamashita *et al*., 1993; Kimura *et al*., 2002). SP that co-express high levels of TCRβ and CD69 are indicative of SP directly post-positive selection. Progressing into a mature state $CD69^+$ T cells upregulate CD62L and downregulate CD69 and reach an egressing phenotype ready to leave the thymus and enter the periphery through the CMJ (Alfonso, McHeyzer-Williams and Rosen, 2006; Bankovich, Shiow and Cyster, 2010; Xing *et al*., 2016). Taken together CD69 and CD62L can distinguish semi-mature T cells from mature egressing thymocytes.

Thus, 7-AAD was used as a marker of cell death, CD45.1 or CD45.2 to determine cell origin (host vs donor) , CD3ε and TCRβ for αβ T cells ($CD3\varepsilon^+TCR\beta^+$), CD25 and CD44 to determine what DN stage T cells are located in DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), DN4 ($CD44^-CD25^-$), CD4 and CD8 for progression from DN to DP and SP stages and CD69 and CD62L to determine semi-mature ($CD69^+CD62L^-$) and mature egressing T cells ($CD69^-CD62L^+$).

Absolute cell counts were calculated for the above cell populations for both host and donor cells. Three independent experiments were carried out for each timepoint. Although some conditions had more than one animal being grafted with the same condition on the same day, grafts recovered from each animal were processed independently and therefore provide separate datapoints, rather than average animals from the same surgical date. As total animal numbers were low and the variability of the iTEC system is high, it was considered the best option to capture the variability seen in these experiments.

iTEC-MultiRTOC, iTEC+FTM-MultiRTOC and positive control RFTOC were grafted into Ly5.1 homozygous mice and recovered after two, four or six weeks *in vivo*. Grafts were removed from under the kidney capsule and mechanically dissociated with a syringe plunger through a 40 μm filter to liberate thymocytes. These were then stained with antibodies mentioned above and analysed on an ACEA Novocyte® cell analyser.

Representative FACS plots for grafts recovered after two, four and six weeks *in vivo* are presented in Figures 5.2-4, Figures 5.5-7 and Figures 5.8-10, respectively.

Absolute cell counts of T cell populations were calculated for each mouse and timepoint and are presented in Tables 5.1- 5.6. A summary of absolute cell count means for each condition and timepoint can be seen in Table 5.7 (donor) and 8 (host).

RFTOC cell numbers are used as a guide for trends over time and for the thymocyte subsets that should be present in an optimal reaggregated transplanted thymic system, rather than for direct comparison of cell numbers, due to differences in the overall cellular inputs in these two systems and because the overall cellularity of the RFTOC was much higher than that of the iTEC RTOC. Due to the latter and the large variability in cell number between experiments, data were normalised through log transformation to view trends over time. Log_{10}(absolute cell number+1) was calculated to ensure the inclusion of any cell populations with an absolute cell count of zero. Bar plots summarising these data for donor T cell subsets present at two, four and six weeks are found in Figure 5.11. Bar plots for host T cell subsets are found in Figure 5.12.

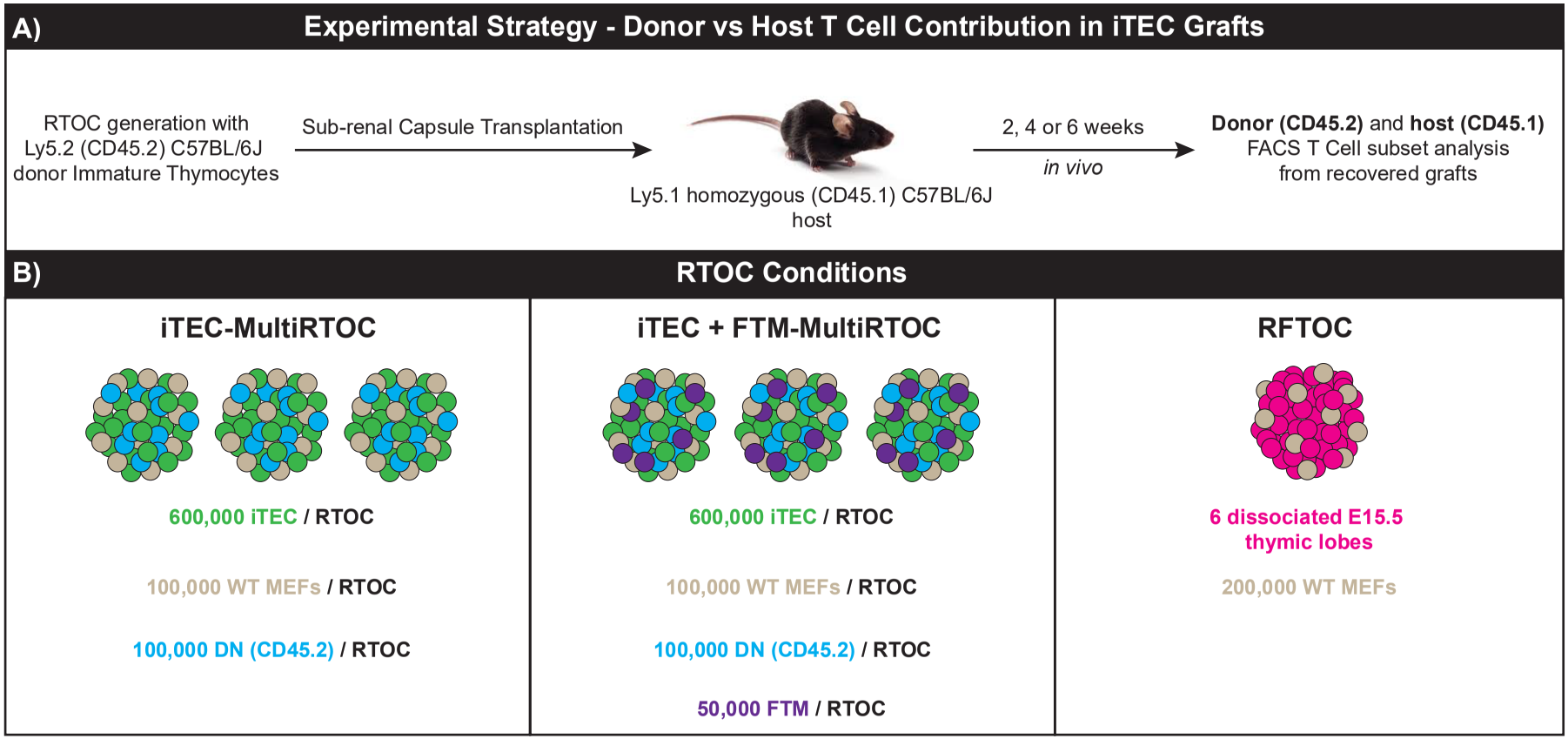
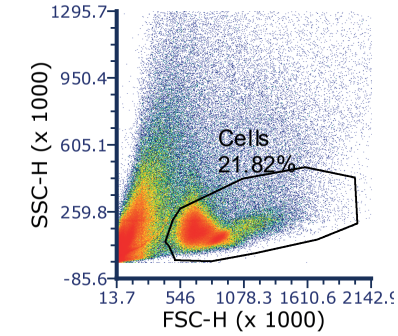
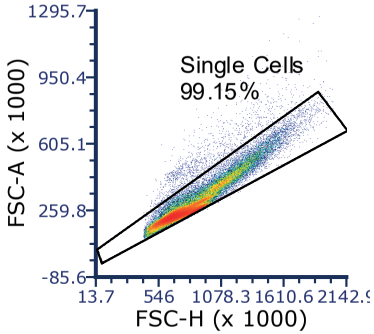
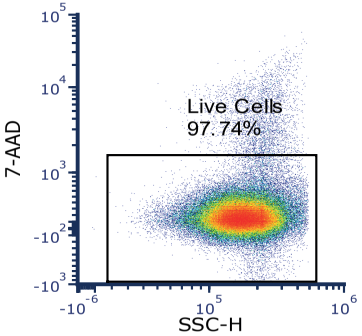
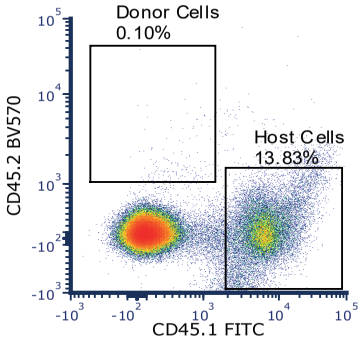


Figure 5.1. Experimental strategy for testing iTEC ability to recruit host lymphoid progenitors and support T Cell lineage commitment and development. A) iTEC are isolated from GFP+ Day 18 iFoxn1 MEFs. Fetal thymic mesenchyme (FTM) and DNs are collected from dissociated E15.5 WT Ly5.2 C57BL/6 thymic lobes as stated previously. WT MEFs are cultured and harvested. RTOC recipients are Ly5.1 homozygous mice on a C57BL/6 background. **B)** RTOC condition components. Detailed description of cells and numbers used in each condition.

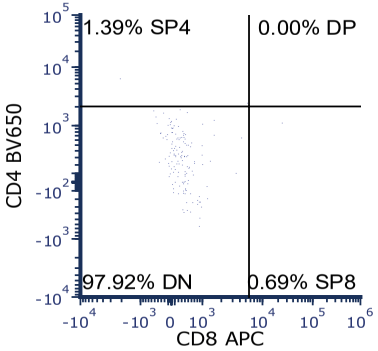


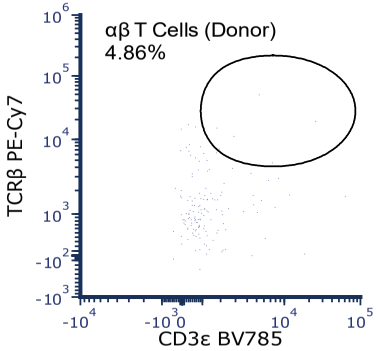




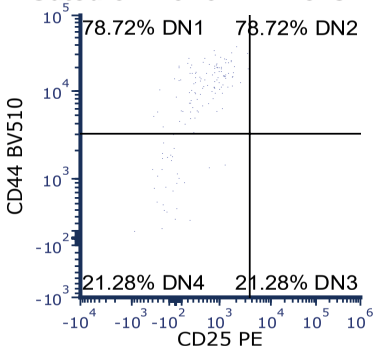


Gated on Donor Cells (CD45.2+)

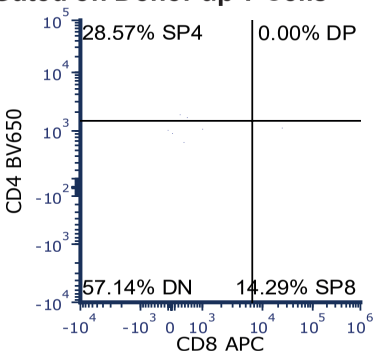




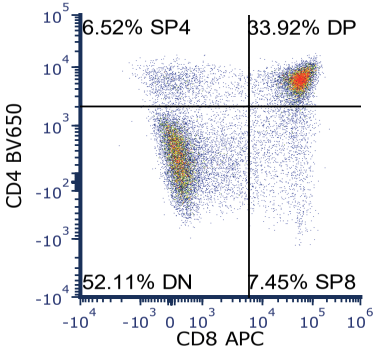
Gated on Donor DN Cells

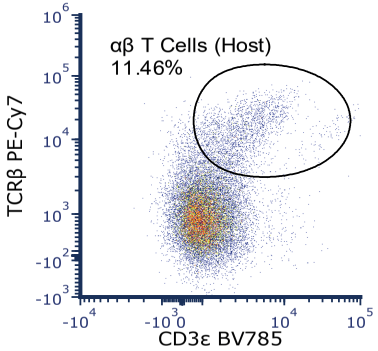


Gated on Donor αβ T Cells



Gated on Host Cells (CD45.1+)





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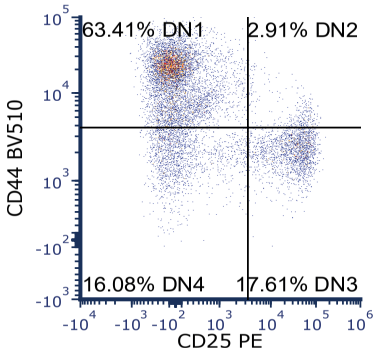
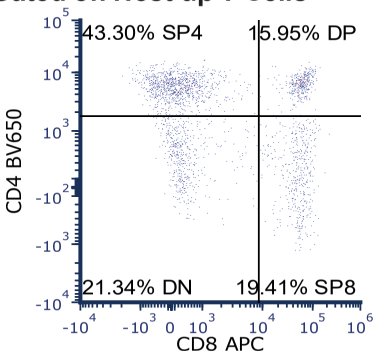


Gated on Host αβ T Cells



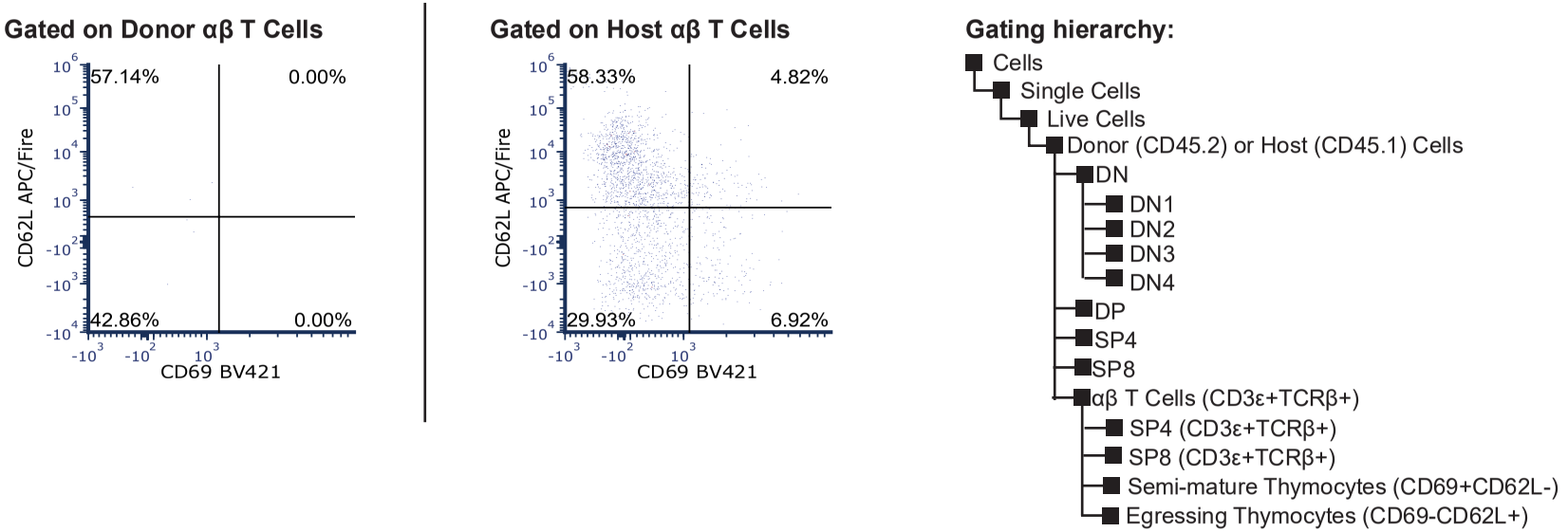
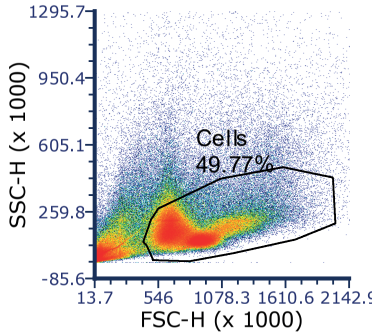
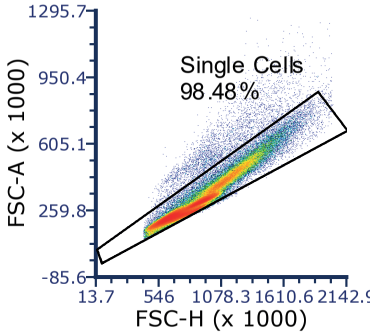
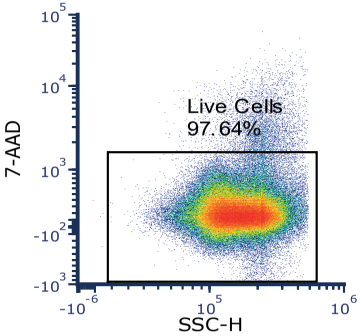
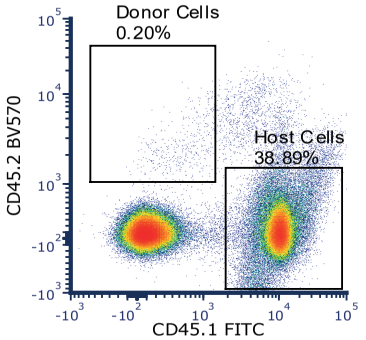


Figure 5.2. Thymopoiesis in iTEC-MultiRTOC grafts recovered 2 weeks post grafting. Representative FACS plots and gating hierarchy for iTEC-MultiRTOC 2 weeks post transplantation. Transplant recipients received three RTOC each consisting of 600,000 iTEC + 100,000 Donor DN Thymocytes +100,000 WT MEFs. Donor and host cells were gated from CD45.2 and CD45.1^{+} cells, respectively. Donor cells were obtained from WT C57BL/6J mice and grafted into host Ly5.1 homozygous C57BL/6J recipients.

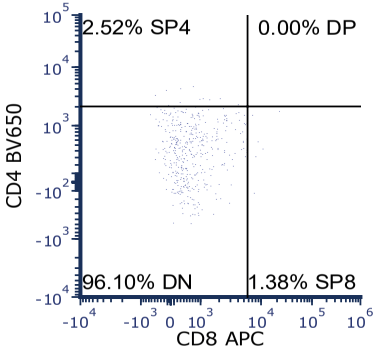


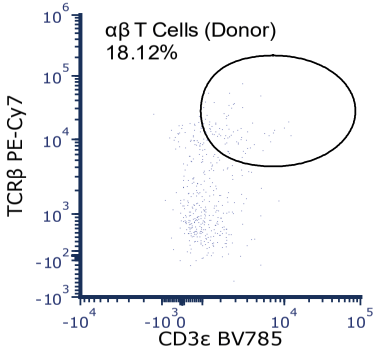




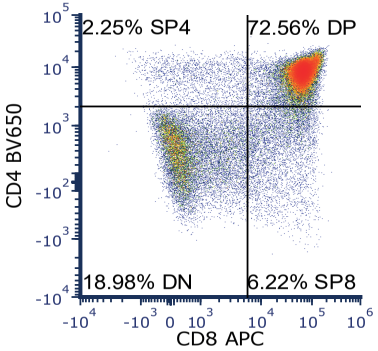


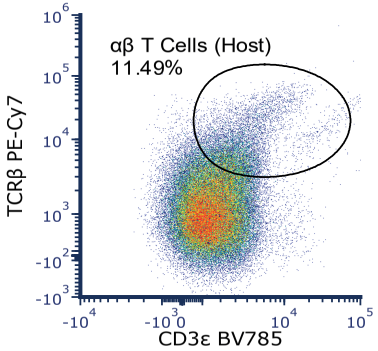
Gated on Donor Cells (CD45.2+)



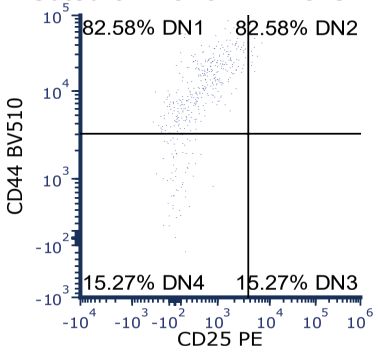


Gated on Host Cells (CD45.1+)

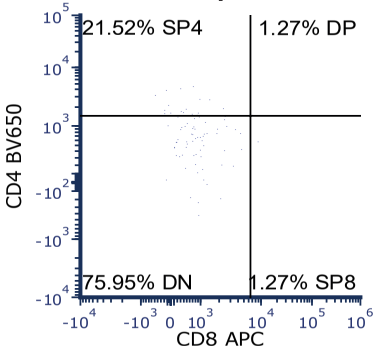




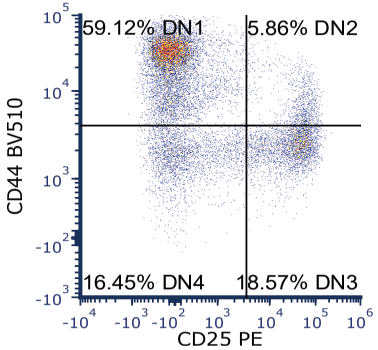
Gated on Donor DN Cells



Gated on Donor αβ T Cells



Gated on Host DN Cells



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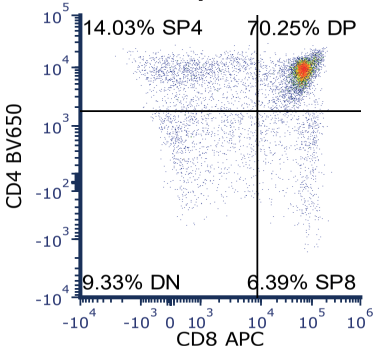


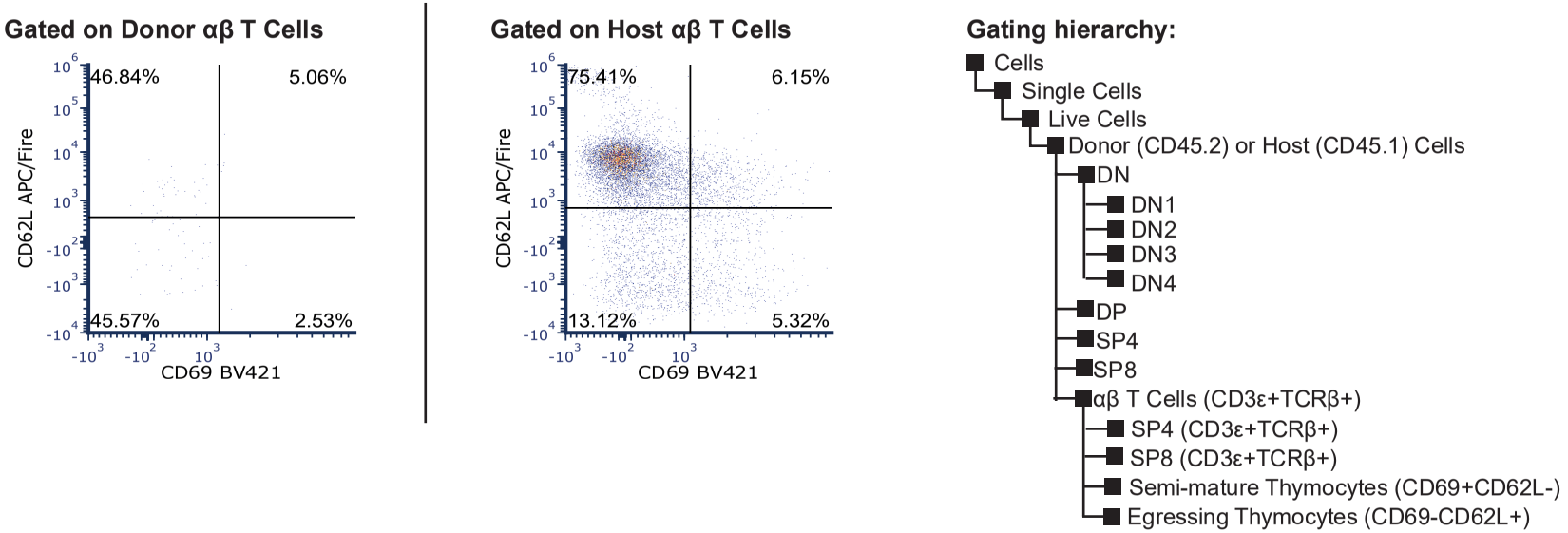
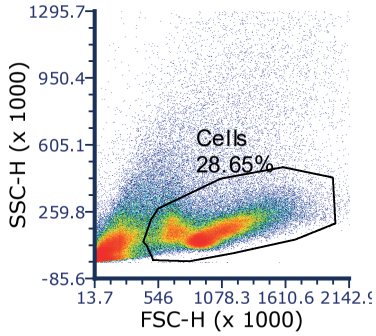
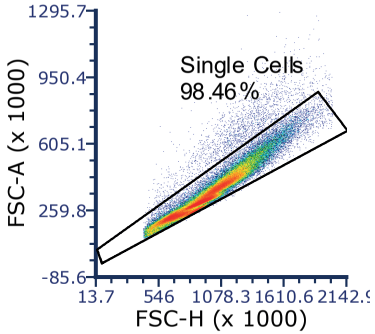
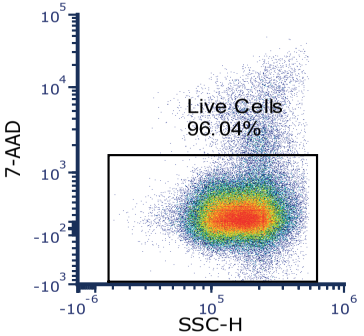
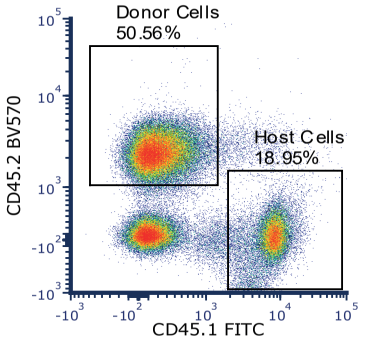


Figure 5.3. Thymopoiesis in iTEC+FTM-MultiRTOC grafts recovered 2 weeks post grafting. Representative FACS plots and gating hierarchy for iTEC+FTM-MultiRTOC 2 weeks post transplantation. Transplant recipients received three RTOC each consisting of 600,000 iTEC + 100,000 Donor DN Thymocytes +100,000 WT MEFs + 50,000 Fetal Thymic Mesenchymal (FTM) cells. Donor and host cells were gated from CD45.2 and CD45.1+ cells, respectively. Donor cells were obtained from WT C57BL/6J mice and grafted into host Ly5.1 homozygous C57BL/6J recipients.

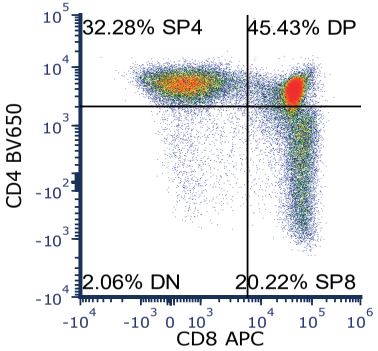


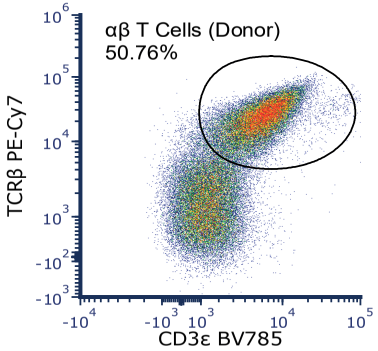




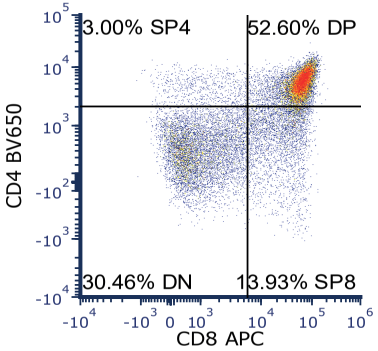


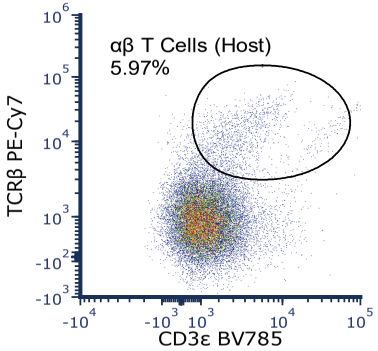
Gated on Donor Cells (CD45.2+)



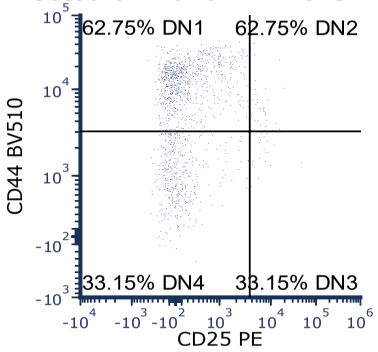


Gated on Host Cells (CD45.1+)

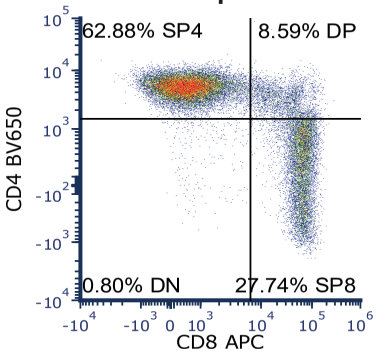




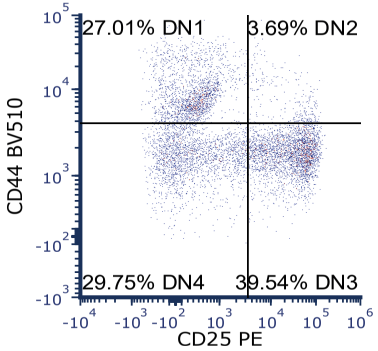
Gated on Donor DN Cells



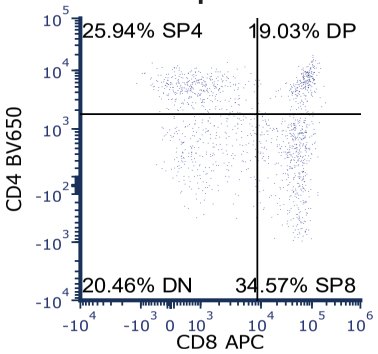
Gated on Donor αβ T Cells



Gated on Host DN Cells



Gated on Host αβ T Cells



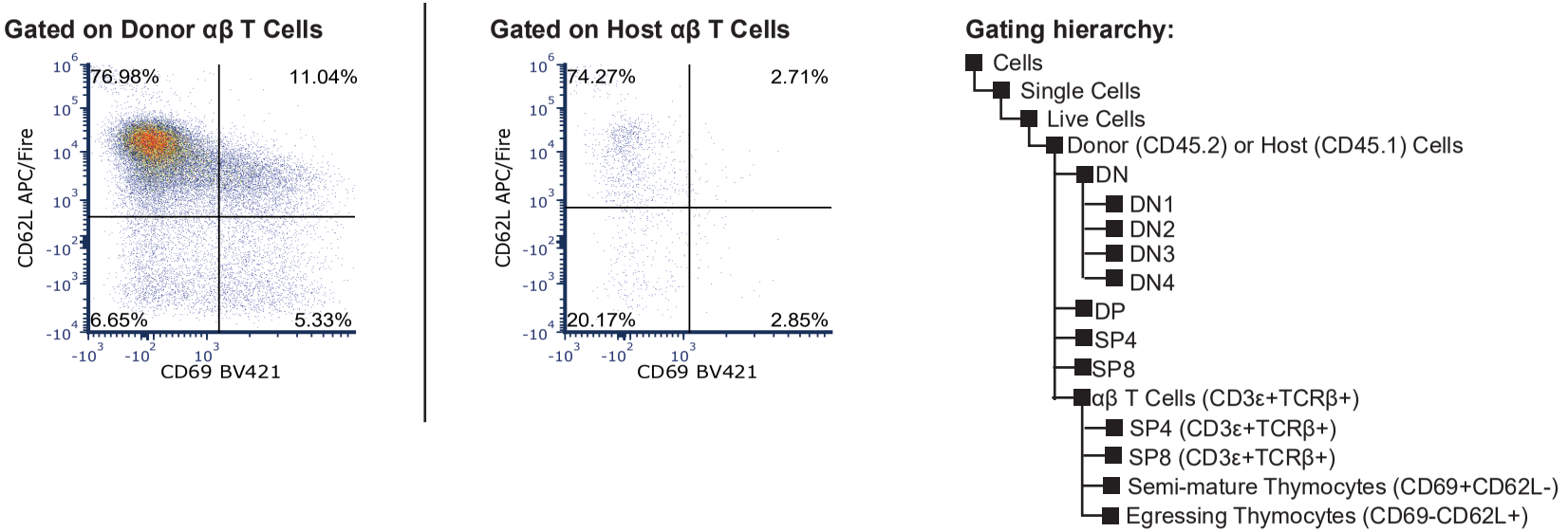


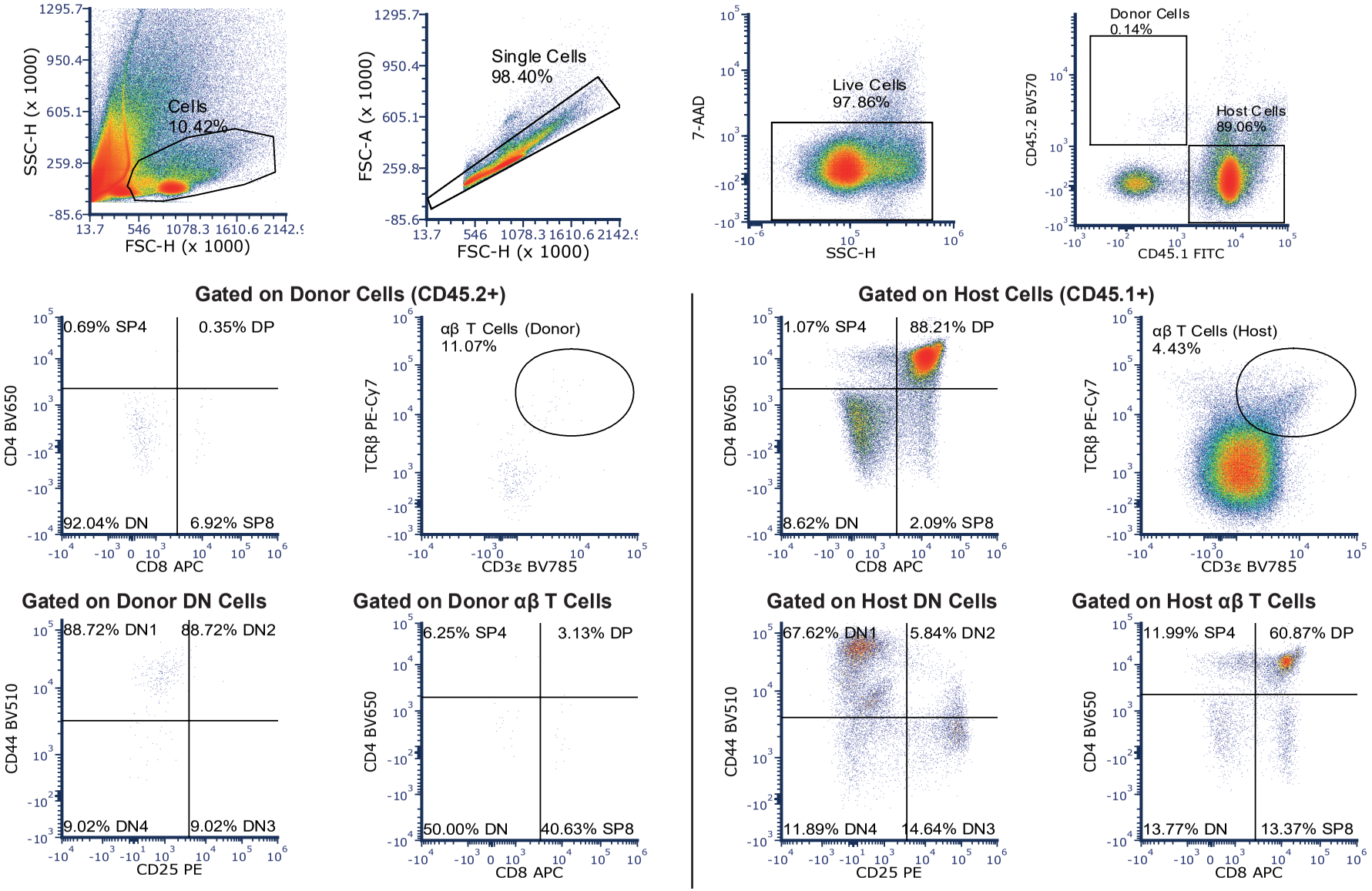
Figure 5.4. Thymopoiesis in RFTOC grafts 2 weeks post grafting. Representative FACS plots and gating hierarchy for RFTOC 2 weeks post transplantation. Transplant recipients received one RFTOC consisting of 6 dissociated whole E15.5 thymic lobes + 200,000 WT MEFs. Donor and host cells were gated from CD45.2 and CD45.1+ cells, respectively. Donor cells were obtained from WT C57BL/6J mice and grafted into host Ly5.1 homozygous C57BL/6J recipients.

Donor, t=2 weeks post-grafting															
Condition	**Animal**	**Donor Cells**	**DN**	**DN1**	**DN2**	**DN3**	**DN4**	**DP**	**SP4**	**SP8**	**CD3ε⁺TCRβ⁺**	**SP4 (CD3ε⁺TCRβ⁺)**	**SP8 (CD3ε⁺TCRβ⁺)**	**Semi-mature**	**Egressing**
iTEC MultiRTOC	1	238	140	84	40	6	10	44	42	12	0	0	0	0	0
	2	52	50	40	2	0	8	0	2	0	10	2	0	0	6
	3	126	104	2	0	0	102	12	10	0	0	0	0	0	0
	4	76	76	14	0	0	62	0	0	0	2	0	0	0	2
	5	462	447	397	0	0	50	0	12	3	97	20	0	4	43
	6	144	141	111	0	0	30	0	2	1	7	2	1	0	4
	Mean	**183 ±138**	**160 ±133**	**108 ±135**	**7 ±15**	**1 ±2**	**44 ±33**	**9 ±16**	**11 ±14**	**3 ±4**	**19 ±35**	**4 ±7**	**0 ±0**	**1 ±1**	**9 ±15**
iTEC+FTM MultiRTOC	1	36	36	18	0	0	18	0	0	0	4	0	0	0	0
	2	436	419	346	9	0	64	0	11	6	79	17	1	2	37
	3	268	246	42	20	2	182	12	10	0	0	0	0	0	0
	Mean	**247 ±164**	**234 ±157**	**135 ±149**	**10 ±8**	**1 ±1**	**88 ±69**	**4 ±6**	**7 ±5**	**2 ±3**	**28 ±36**	**6 ±8**	**0 ±0**	**1 ±1**	**12 ±17**
RFTOC	1	145354	536	210	66	28	218	138982	3216	2620	124	84	6	88	2
	2	62718	1304	814	34	21	435	28430	20249	12735	31833	20031	8804	1685	24519
	3	434004	2638	622	30	32	1954	369678	32010	29678	59284	31644	15330	7770	36060
	4	33740	474	94	2	2	376	25354	4642	3270	8106	4610	2326	760	5664
	Mean	**168954 ± 158421**	**1238 ± 872**	**435 ± 294**	**33 ±23**	**21 ±12**	**746 ±702**	**140611 ± 139949**	**15029 ± 11864**	**12076 ±10923**	**24837 ±23055**	**14092 ±12545**	**6617 ±5975**	**2576 ±3052**	**16561 ±14461**

Table 5.1. Absolute cell counts of donor cell populations in recovered grafts at two weeks *in vivo*. Detailed account of absolute cell numbers obtained from each technical replicate along with mean ±SD per condition.

Host, t= 2 weeks post-grafting															
Condition	**Animal**	**Host Cells**	**DN**	**DN1**	**DN2**	**DN3**	**DN4**	**DP**	**SP4**	**SP8**	**CD3ε⁺TCRβ⁺**	**SP4 (CD3ε⁺TCRβ⁺)**	**SP8 (CD3ε⁺TCRβ⁺)**	**Semi-mature**	**Egressing**
iTEC MultiRTOC	1	13722	930	512	216	128	74	11122	122	1548	10	0	0	4	0
	2	3918	2826	2350	28	130	318	632	276	184	1044	242	108	168	400
	3	5946	4442	3346	986	28	82	64	376	1064	56	18	4	24	6
	4	4776	4130	3666	12	38	414	258	224	164	752	200	126	74	360
	5	48221	14740	6021	1548	4007	3164	28427	1240	3814	3439	661	369	165	2418
	6	19912	10382	6573	294	1831	1684	6747	1297	1486	2282	988	444	156	1333
	Mean	**16083 ± 15447**	**6242 ± 4777**	**3745 ± 2071**	**514 ± 565**	**1027 ± 1478**	**956 ± 1129**	**7875 ± 10047**	**589 ± 486**	**1377 ± 1223**	**1264 ± 1232**	**352 ± 358**	**175 ± 172**	**99 ± 68**	**753 ± 867**
iTEC+FTM MultiRTOC	1	3802	3062	2556	16	52	438	426	198	116	680	194	78	56	356
	2	83792	15923	9404	915	2976	2628	60773	1880	5216	9625	1350	615	512	7262
	3	4374	2398	1322	500	486	90	282	180	1514	18	8	2	12	0
	Mean	**30656 ± 37574**	**7128 ± 6225**	**4427 ± 3555**	**477 ± 367**	**1171 ± 1288**	**1052 ± 1123**	**20494 ± 28482**	**753 ± 797**	**2282 ± 2152**	**3441 ± 4381**	**517 ± 594**	**232 ± 273**	**193 ± 226**	**2539 ± 3343**
RFTOC	1	8150	1374	204	70	1038	62	3618	224	2934	8	2	2	2	0
	2	23500	7170	1931	256	2834	2149	12344	706	3280	1403	361	485	40	1043
	3	13770	2752	1356	78	532	786	9028	702	1288	1906	622	634	134	1214
	4	49652	2284	370	10	1086	818	45352	326	1690	2174	244	164	90	1644
	Mean	**23768 ± 15921**	**3395 ± 2235**	**965 ± 710**	**104 ± 92**	**1373 ± 871**	**954 ± 753**	**17586 ± 16331**	**490 ± 218**	**2298 ± 830**	**1373 ± 835**	**307 ± 223**	**321 ± 251**	**67 ± 50**	**975 ± 604**

Table 5.2. Absolute cell counts of host cell populations in recovered grafts at two weeks *in vivo*. Detailed account of absolute cell numbers obtained from each technical replicate along with mean ±SD per condition.


Cells
10.42%
SSC-H (x 1000)
FSC-H (x 1000)
Single Cells
98.40%
FSC-A (x 1000)
Live Cells
97.86%
7-AAD
SSC-H
Donor Cells
0.14%
Host Cells
89.06%
CD45.2 BV570
CD45.1 FITC
Gated on Donor Cells (CD45.2+)
Gated on Host Cells (CD45.1+)
0.69% SP4
0.35% DP
92.04% DN
6.92% SP8
CD4 BV650
CD8 APC
αβ T Cells (Donor)
11.07%
TCRβ PE-Cy7
CD3ε BV785
1.07% SP4
88.21% DP
8.62% DN
2.09% SP8
αβ T Cells (Host)
4.43%
Gated on Donor DN Cells
Gated on Donor αβ T Cells
Gated on Host DN Cells
Gated on Host αβ T Cells
88.72% DN1
88.72% DN2
9.02% DN4
9.02% DN3
CD44 BV510
CD25 PE
6.25% SP4
3.13% DP
50.00% DN
40.63% SP8
67.62% DN1
5.84% DN2
11.89% DN4
14.64% DN3
11.99% SP4
60.87% DP
13.77% DN
13.37% SP8

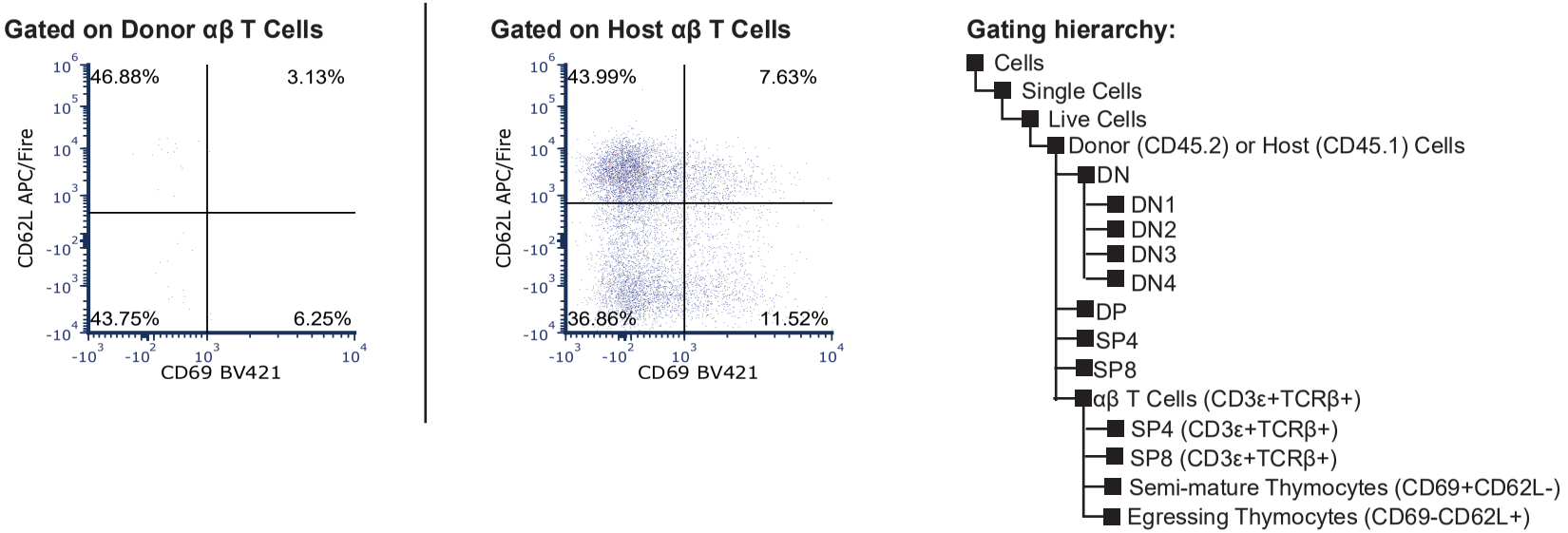
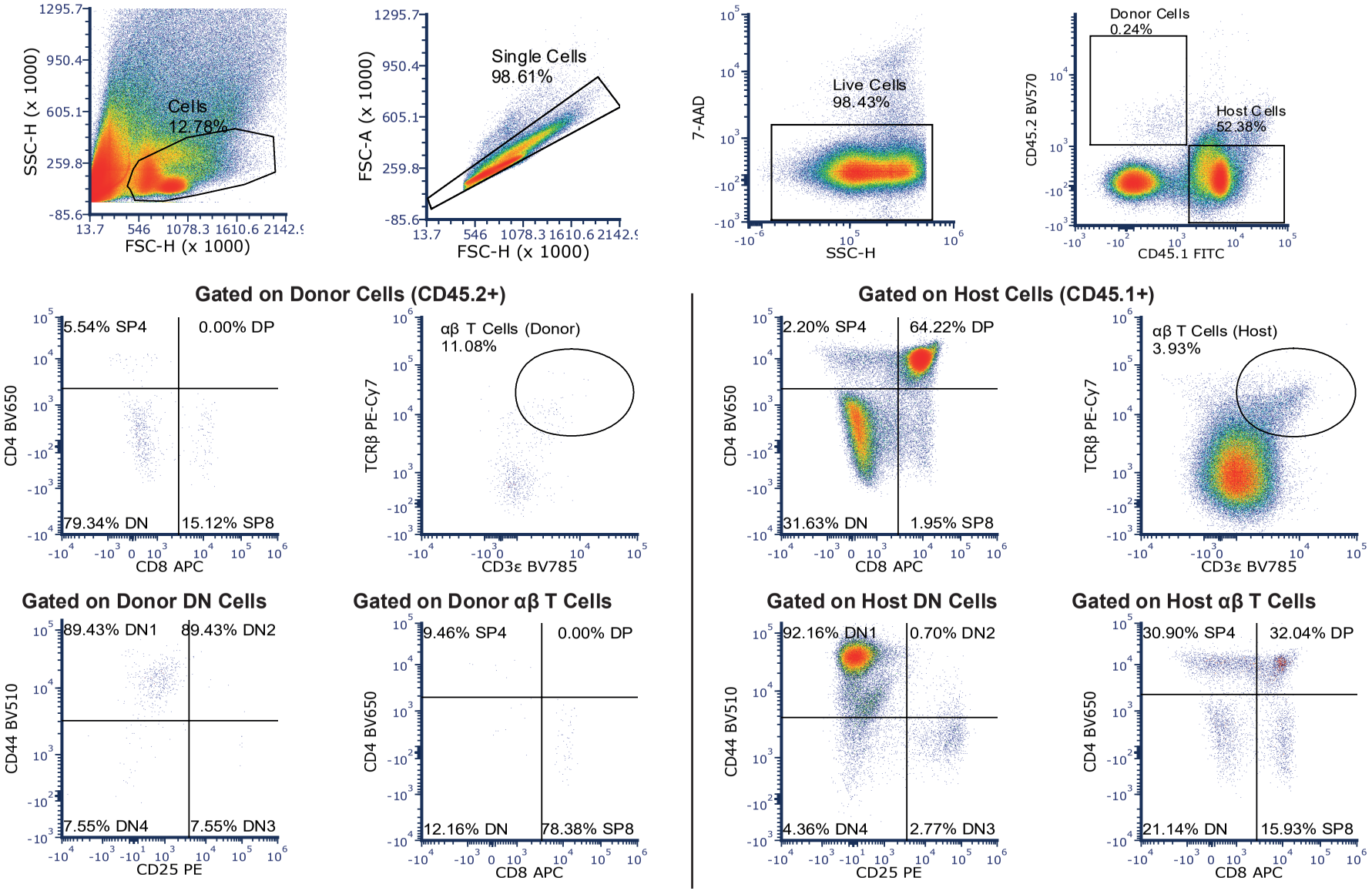


Figure 5.5. Thymopoiesis in iTEC-MultiRTOC 4 weeks post grafting. Representative FACS plots and gating hierarchy for iTEC-MultiRTOC 4 weeks post transplantation. Transplant recipients received three RTOC each consisting of 600,000 iTEC + 100,000 Donor DN Thymocytes +100,000 WT MEFs. Donor and host cells were gated from CD45.2 and CD45.1+ cells, respectively. Donor cells were obtained from WT C57BL/6J mice and grafted into host Ly5.1 homozygous C57BL/6J recipients.


Cells
12.78%
SSC-H (x 1000)
FSC-H (x 1000)
Single Cells
98.61%
FSC-A (x 1000)
FSC-H (x 1000)
Live Cells
98.43%
7-AAD
SSC-H
Donor Cells
0.24%
Host Cells
52.38%
CD45.2 BV570
CD45.1 FITC
Gated on Donor Cells (CD45.2+)
5.54% SP4
0.00% DP
79.34% DN
15.12% SP8
CD4 BV650
CD8 APC
αβ T Cells (Donor)
11.08%
TCRβ PE-Cy7
CD3ε BV785
Gated on Host Cells (CD45.1+)
2.20% SP4
64.22% DP
31.63% DN
1.95% SP8
CD4 BV650
CD8 APC
αβ T Cells (Host)
3.93%
TCRβ PE-Cy7
CD3ε BV785
Gated on Donor DN Cells
89.43% DN1
89.43% DN2
7.55% DN4
7.55% DN3
CD44 BV510
CD25 PE
Gated on Donor αβ T Cells
9.46% SP4
0.00% DP
12.16% DN
78.38% SP8
CD4 BV650
CD8 APC
Gated on Host DN Cells
92.16% DN1
0.70% DN2
4.36% DN4
2.77% DN3
CD44 BV510
CD25 PE
Gated on Host αβ T Cells
30.90% SP4
32.04% DP
21.14% DN
15.93% SP8
CD4 BV650
CD8 APC

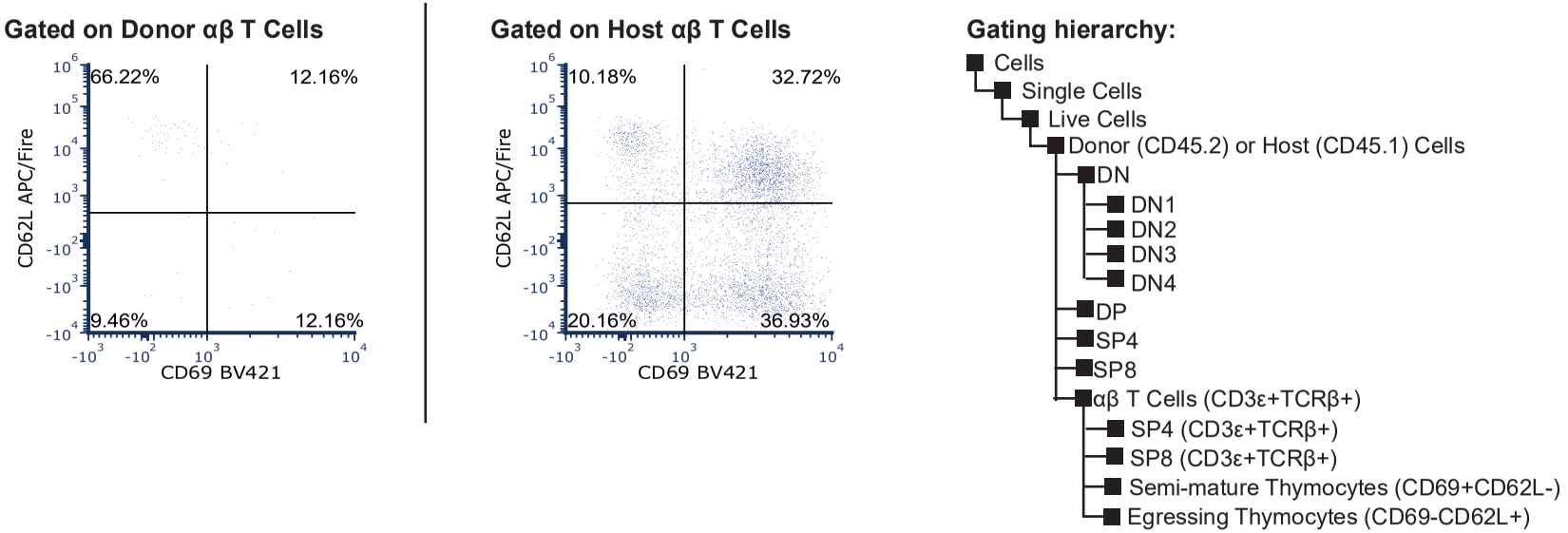
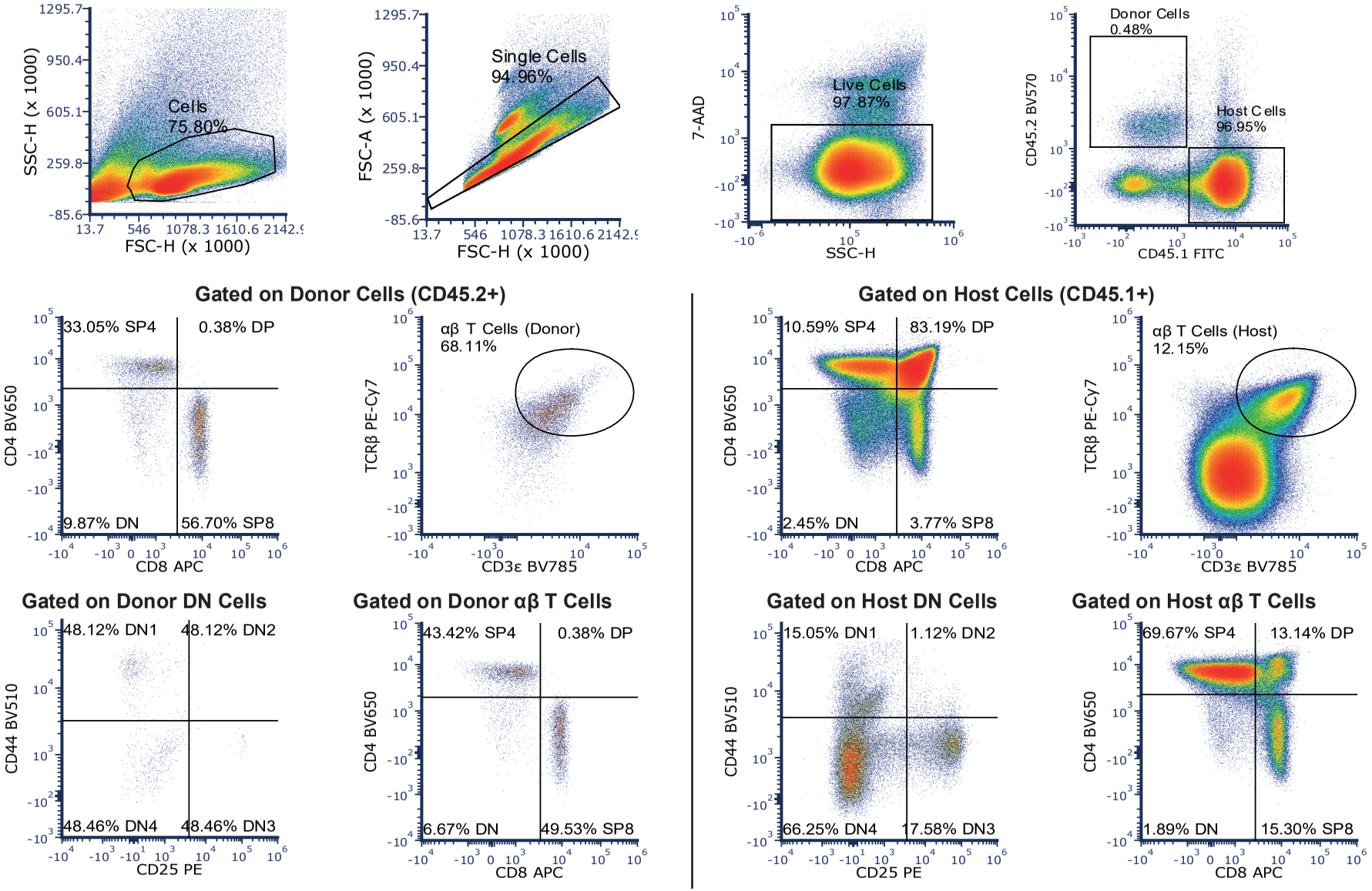


Figure 5.6. Thymopoiesis in iTEC+FTM-MultiRTOC grafts 4 weeks post grafting. Representative FACS plots and gating hierarchy for iTEC+FTM-MultiRTOC 4 weeks post transplantation. Transplant recipients received three RTOC each consisting of 600,000 iTEC + 100,000 Donor DN Thymocytes +100,000 WT MEFs + 50,000 Fetal Thymic Mesenchymal cells. Donor and host cells were gated from CD45.2 and CD45.1+ cells, respectively. Donor cells were obtained from WT C57BL/6J mice and grafted into host Ly5.1 homozygous C57BL/6J recipients.


Cells
75.80%
SSC-H (x 1000)
FSC-H (x 1000)
Single Cells
94.96%
FSC-A (x 1000)
FSC-H (x 1000)
Live Cells
97.87%
7-AAD
SSC-H
Donor Cells
0.48%
Host Cells
96.95%
CD45.2 BV570
CD45.1 FITC
Gated on Donor Cells (CD45.2+)
33.05% SP4
0.38% DP
9.87% DN
56.70% SP8
CD4 BV650
CD8 APC
αβ T Cells (Donor)
68.11%
TCRβ PE-Cy7
CD3ε BV785
Gated on Host Cells (CD45.1+)
10.59% SP4
83.19% DP
2.45% DN
3.77% SP8
CD4 BV650
CD8 APC
αβ T Cells (Host)
12.15%
TCRβ PE-Cy7
CD3ε BV785
Gated on Donor DN Cells
48.12% DN1
48.12% DN2
48.46% DN4
48.46% DN3
CD44 BV510
CD25 PE
Gated on Donor αβ T Cells
43.42% SP4
0.38% DP
6.67% DN
49.53% SP8
CD4 BV650
CD8 APC
Gated on Host DN Cells
15.05% DN1
1.12% DN2
66.25% DN4
17.58% DN3
CD44 BV510
CD25 PE
Gated on Host αβ T Cells
69.67% SP4
13.14% DP
1.89% DN
15.30% SP8
CD4 BV650
CD8 APC

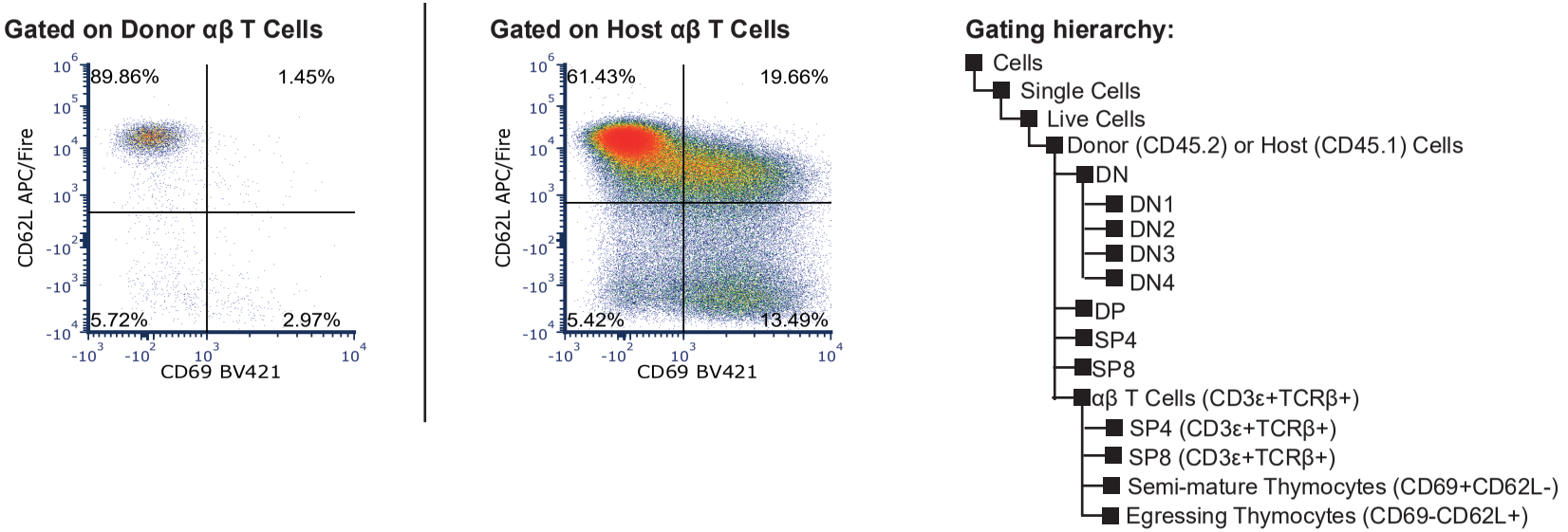


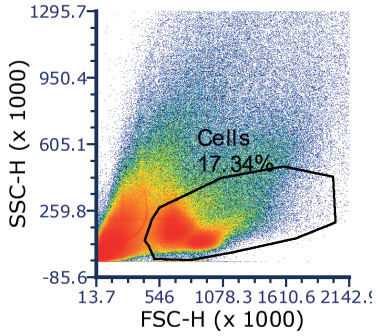
Figure 5.7. Thymopoiesis in RFTOC grafts 4 weeks post grafting. Representative FACS plots and gating hierarchy for RFTOC 4 weeks post transplantation. Transplant recipients received one RFTOC consisting of 6 dissociated whole E15.5 thymic lobes + 200,000 WT MEFs. Donor and host cells were gated from CD45.2 and CD45.1+ cells, respectively. Donor cells were obtained from WT C57BL/6J mice and grafted into host Ly5.1 homozygous C57BL/6J recipients.

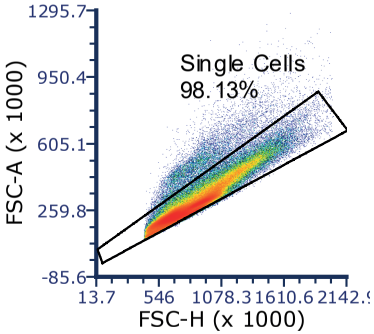
Donor, t=4 weeks post-grafting															
Condition	**Animal**	**Donor Cells**	**DN**	**DN1**	**DN2**	**DN3**	**DN4**	**DP**	**SP4**	**SP8**	**CD3ε⁺TCRβ⁺**	**SP4 (CD3ε⁺TCRβ⁺)**	**SP8 (CD3ε⁺TCRβ⁺)**	**Semi-mature**	**Egressing**
iTEC MultiRTOC	1	94	84	70	0	0	14	0	10	0	9	1	0	1	3
	2	10	8	4	0	0	4	0	2	0	1	0	0	0	0
	3	116	93	21	1	0	71	1	22	0	26	8	0	3	7
	4	69	54	21	0	0	33	0	15	0	2	1	0	0	0
	5	289	266	236	6	0	24	0	3	20	32	2	13	2	15
	Mean	**116 ± 94**	**101 ± 88**	**70 ± 86**	**1 ± 2**	**0 ± 0**	**29 ± 23**	**0 ± 0**	**10 ± 7**	**4 ± 8**	**14 ± 13**	**2 ± 3**	**3 ± 5**	**1 ± 1**	**5 ± 6**
iTEC+FTM MultiRTOC	1	20	6	4	0	0	2	0	14	0	0	0	0	0	0
	2	119	95	79	0	0	16	0	10	14	10	2	3	0	5
	3	668	531	474	10	6	41	0	37	100	74	7	58	9	49
	Mean	**269 ± 285**	**211 ± 229**	**186 ± 206**	**3 ± 5**	**2 ± 3**	**20 ± 16**	**0 ± 0**	**20 ± 12**	**38 ± 44**	**28 ± 33**	**3 ± 3**	**20 ± 27**	**3 ± 4**	**18 ± 22**
RFTOC	1	9905	971	704	30	52	185	546	3888	4500	8571	3643	3927	146	7470
	2	2645	811	362	4	2	443	54	545	1235	1361	382	512	113	786
	3	1739	107	69	2	0	36	48	506	1078	1164	425	674	9	1106
	4	8887	878	423	3	27	425	36	2934	5039	6053	2623	3000	180	5439
	Mean	**5794 ± 3634**	**692 ± 342**	**390 ± 226**	**10 ± 12**	**20 ± 21**	**272 ± 170**	**171 ± 217**	**1968 ± 1482**	**2963 ± 1817**	**4287 ± 3154**	**1768 ± 1412**	**2028 ± 1473**	**112 ± 64**	**3700 ± 2849**

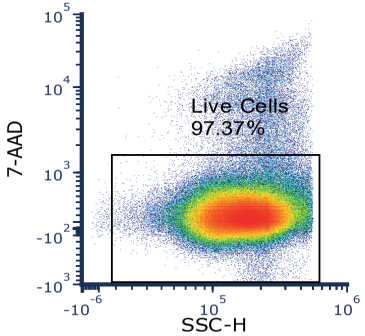
Table 5.3. Absolute cell counts of donor cell populations in recovered grafts at four weeks *in vivo*. Detailed account of absolute cell numbers obtained from each technical replicate along with mean ±SD per condition.

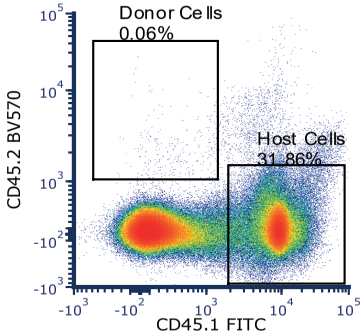
Host, t=4 weeks post-grafting															
Condition	**Animal**	**Host Cells**	**DN**	**DN1**	**DN2**	**DN3**	**DN4**	**DP**	**SP4**	**SP8**	**CD3ε⁺TCRβ⁺**	**SP4 (CD3ε⁺TCRβ⁺)**	**SP8 (CD3ε⁺TCRβ⁺)**	**Semi-mature**	**Egressing**
iTEC MultiRTOC	1	36634	13118	11219	115	113	1671	19153	2466	1897	3776	1598	619	148	1629
	2	4989	4257	3884	0	0	373	4	636	92	706	385	55	59	226
	3	26540	7231	4663	20	172	2376	14255	3258	1796	3985	2278	860	394	1470
	4	53886	7141	3494	74	1029	2544	44331	1470	944	1235	619	128	104	568
	5	177754	15409	10415	892	2255	1847	156445	2255	3645	7881	1097	1039	908	3467
	Mean	**59961 ± 60984**	**9431 ± 4152**	**6735 ± 3364**	**220 ± 338**	**714 ± 853**	**1762 ± 766**	**46838 ± 56642**	**2017 ± 895**	**1675 ± 1182**	**3517 ± 2547**	**1195 ± 683**	**540 ± 391**	**323 ± 315**	**1472 ± 1129**
iTEC+FTM MultiRTOC	1	7297	6698	5901	0	0	797	3	453	143	432	167	55	18	176
	2	101868	9480	3748	1201	2385	2146	81870	5238	5280	8260	3322	1752	1422	3260
	3	148836	47153	43420	326	1318	2089	94802	4052	2829	5852	1982	927	2161	596
	Mean	**86000 ± 58862**	**21110 ± 18450**	**17690 ± 18215**	**509 ± 507**	**1234 ± 975**	**1677 ± 623**	**58892 ± 41974**	**3248 ± 2035**	**2751 ± 2098**	**4848 ± 3274**	**1824 ± 1293**	**911 ± 693**	**1200 ± 889**	**1344 ± 1366**
RFTOC	1	1344426	25621	1985	144	4069	19423	1064497	168212	86096	291053	159995	43980	32297	140271
	2	1133322	51897	6939	657	8982	35319	784103	141537	155785	179930	115362	34578	24731	109973
	3	762887	13448	3541	165	1572	8170	638525	70551	40363	104737	66152	19446	10444	65712
	4	1805657	44225	6627	496	7774	29328	1502172	191067	68193	219375	152991	33561	29583	134764
	Mean	**1261573 ± 376838**	**33798 ± 15143**	**4773 ± 2087**	**366 ± 219**	**5599 ± 2947**	**23060 ± 10302**	**997324 ± 329235**	**142842 ± 45269**	**87609 ± 42601**	**198774 ± 67338**	**123625 ± 37271**	**32891 ± 8761**	**24264 ± 8427**	**112680 ± 29421**

Table 5.4. Absolute cell counts of host cell populations in recovered grafts at four weeks *in vivo*. Detailed account of absolute cell numbers obtained from each technical replicate along with mean ±SD per condition.

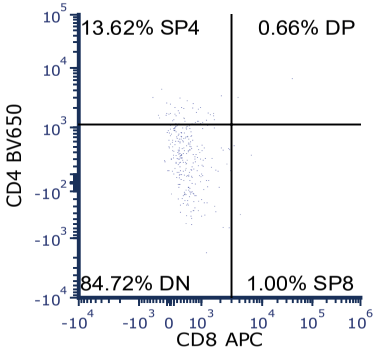


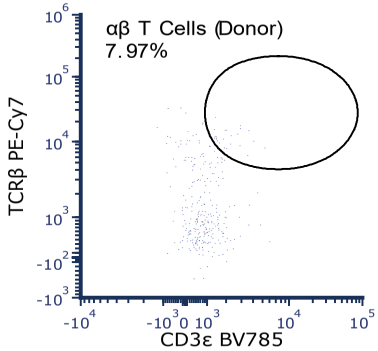




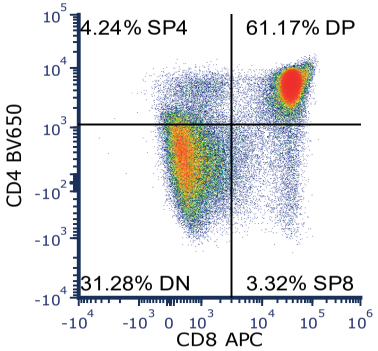


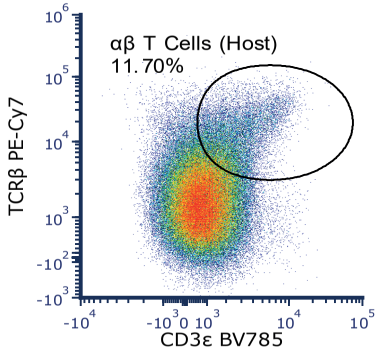
Gated on Donor Cells (CD45.2+)



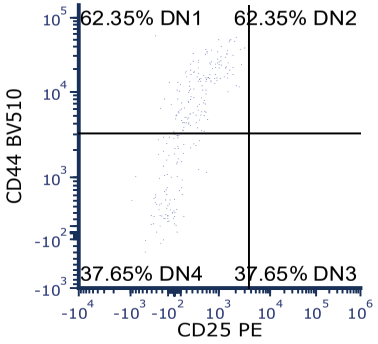


Gated on Host Cells (CD45.1+)

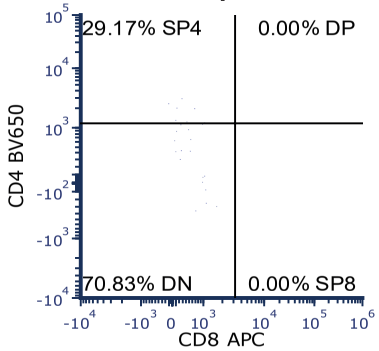




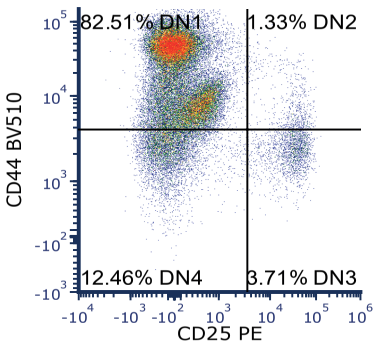
Gated on Donor DN Cells



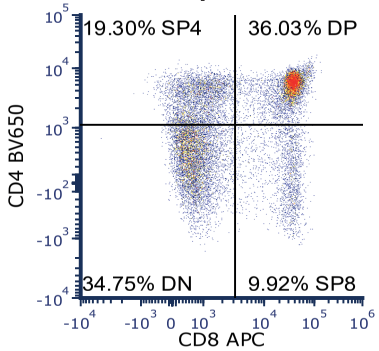
Gated on Donor αβ T Cells



Gated on Host DN Cells



Gated on Host αβ T Cells



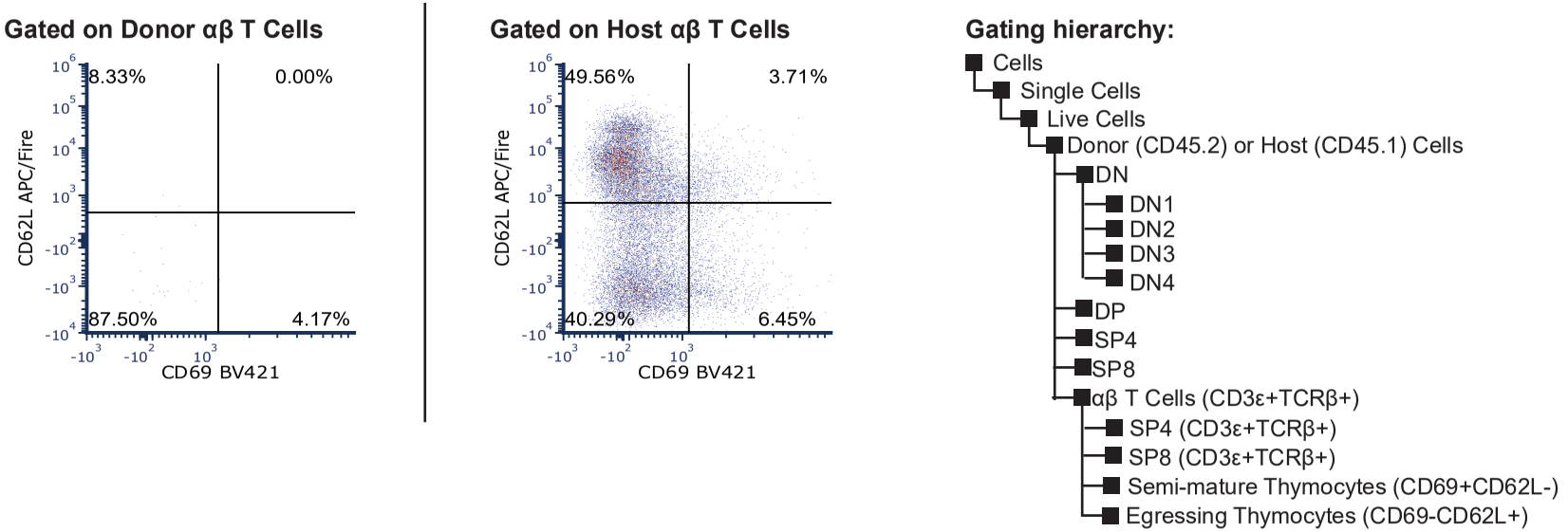
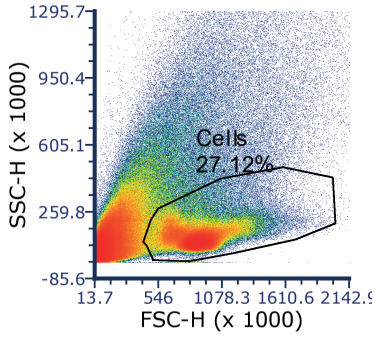
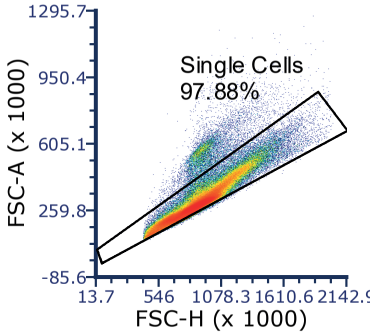
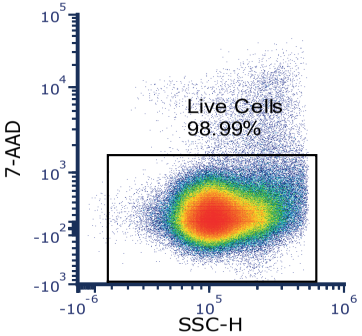
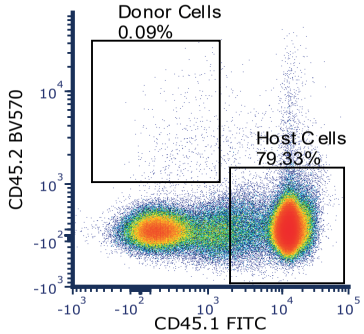


Figure 5.8. Thymopoiesis in iTEC-MultiRTOC grafts 6 weeks post grafting. Representative FACS plots and gating hierarchy for iTEC MultiRTOC 6 weeks post transplantation. Transplant recipients received three RTOC each consisting of 600,000 iTEC + 100,000 Donor DN Thymocytes +100,000 WT MEFs. Donor and host cells were gated from CD45.2 and CD45.1+ cells, respectively. Donor cells were obtained from WT C57BL/6J mice and grafted into host Ly5.1 homozygous C57BL/6J recipients.

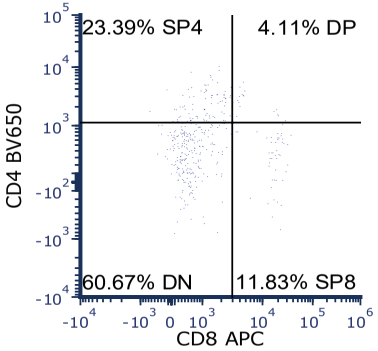


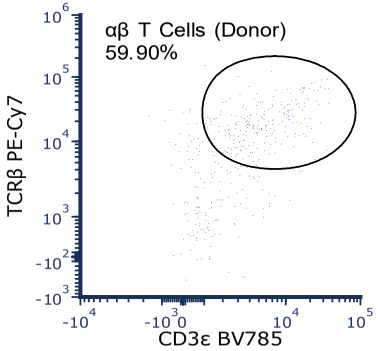




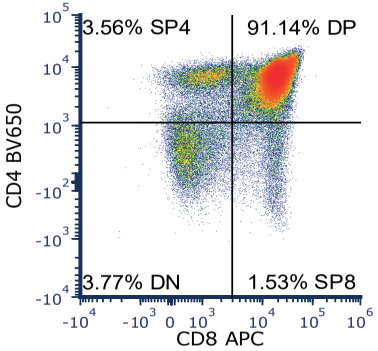


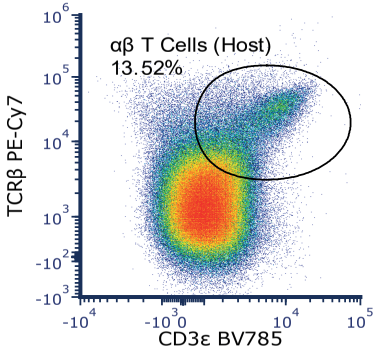
Gated on Donor Cells (CD45.2+)



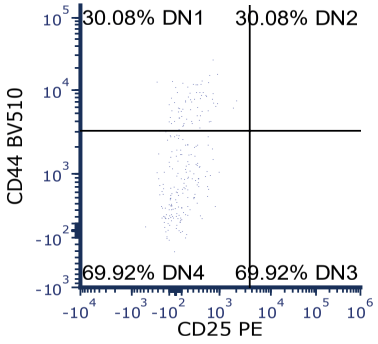


Gated on Host Cells (CD45.1+)

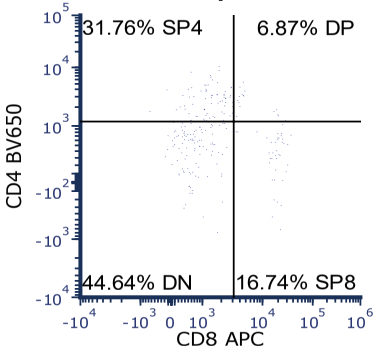




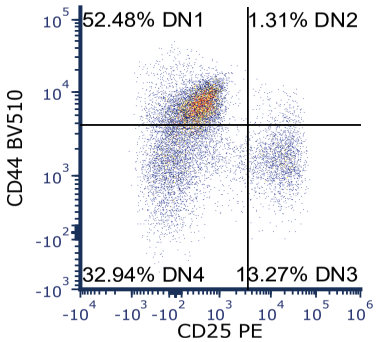
Gated on Donor DN Cells



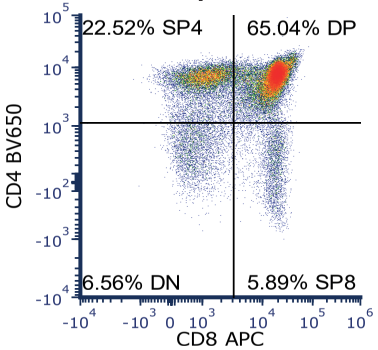
Gated on Donor αβ T Cells



Gated on Host DN Cells



Gated on Host αβ T Cells



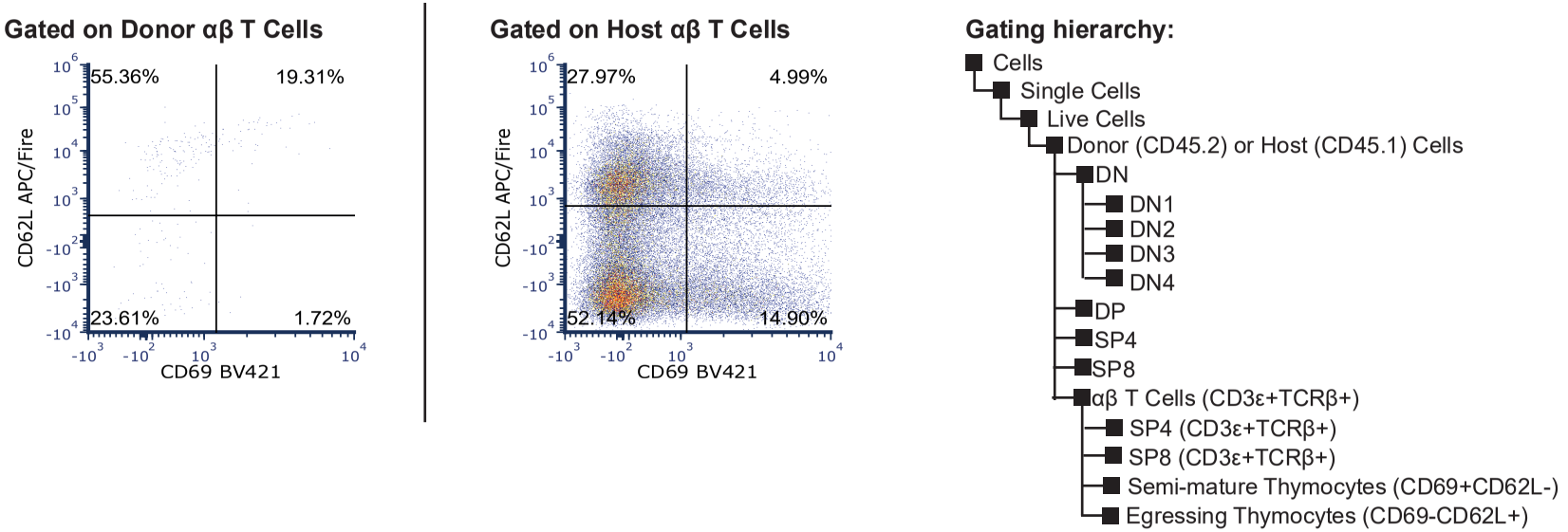
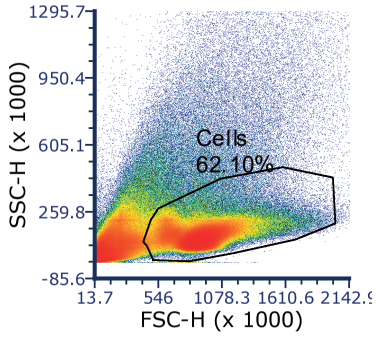
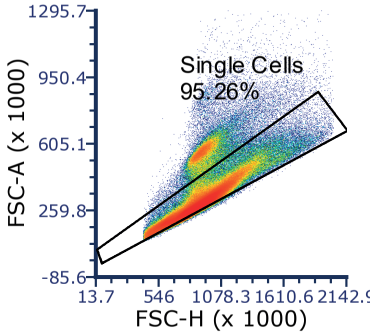
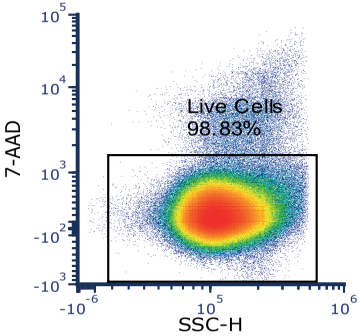
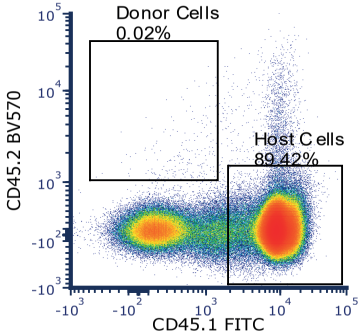


Figure 5.9. Thymopoiesis in iTEC+FTM-MultiRTOC grafts 6 weeks post grafting. Representative FACS plots and gating hierarchy for iTEC +FTM-MultiRTOC 6 weeks post transplantation. Transplant recipients received three RTOC each consisting of 600,000 iTEC + 100,000 Donor DN Thymocytes +100,000 WT MEFs + 50,000 Fetal Thymic Mesenchymal (FTM) cells. Donor and host cells were gated from CD45.2 and CD45.1+ cells, respectively. Donor cells were obtained from WT C57BL/6J mice and grafted into host Ly5.1 homozygous C57BL/6J recipients.

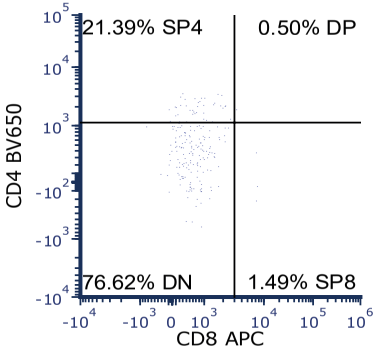


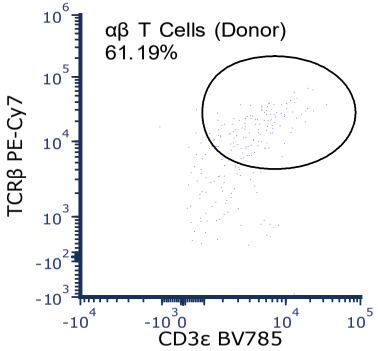




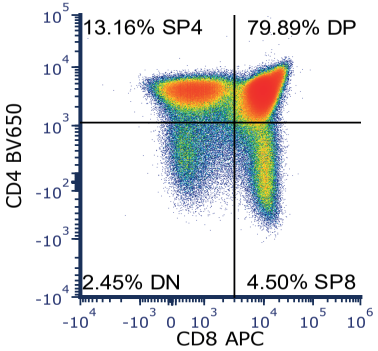


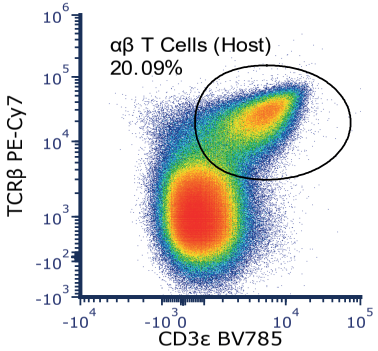
Gated on Donor Cells (CD45.2+)



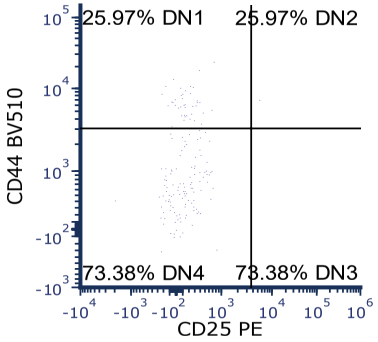


Gated on Host Cells (CD45.1+)

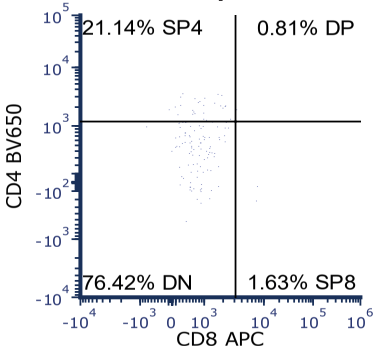




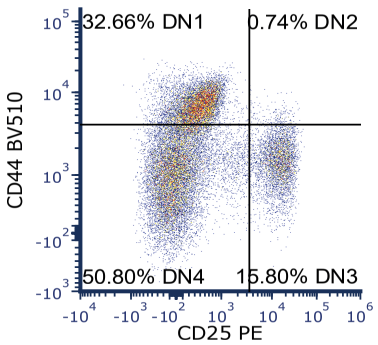
Gated on Donor DN Cells



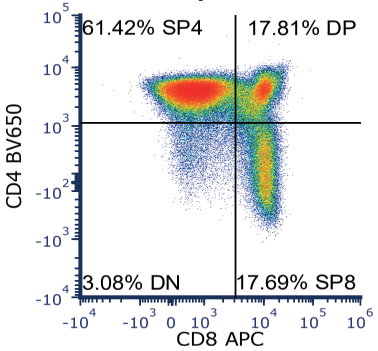
Gated on Donor αβ T Cells



Gated on Host DN Cells



Gated on Host αβ T Cells



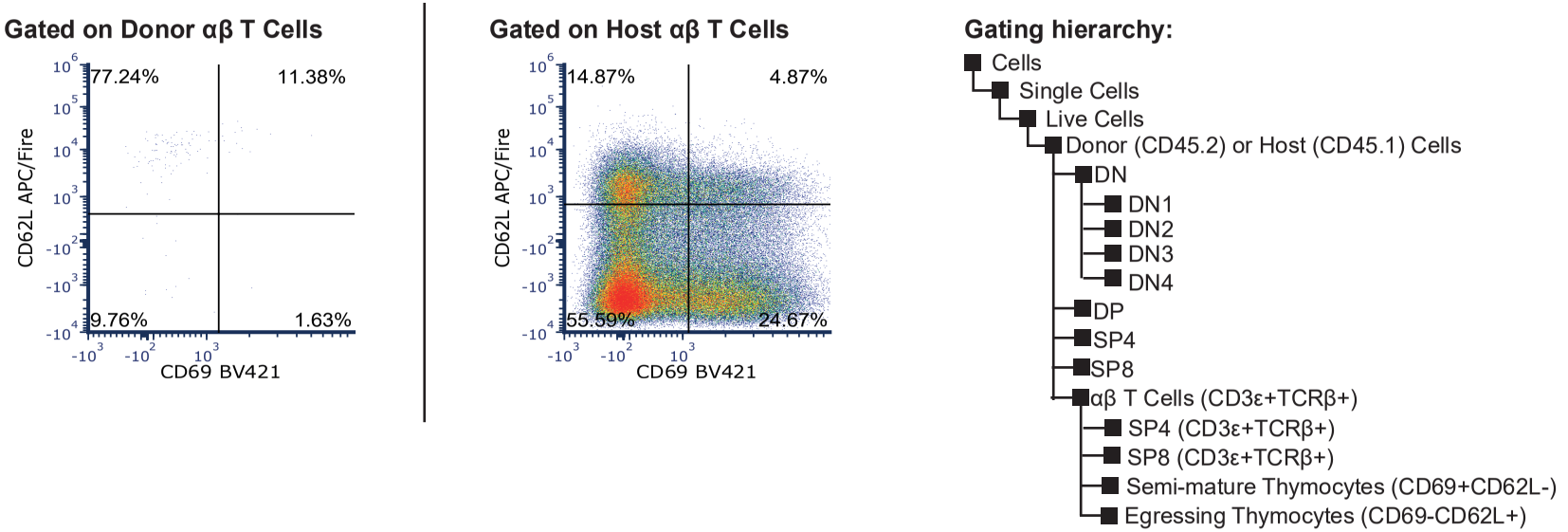


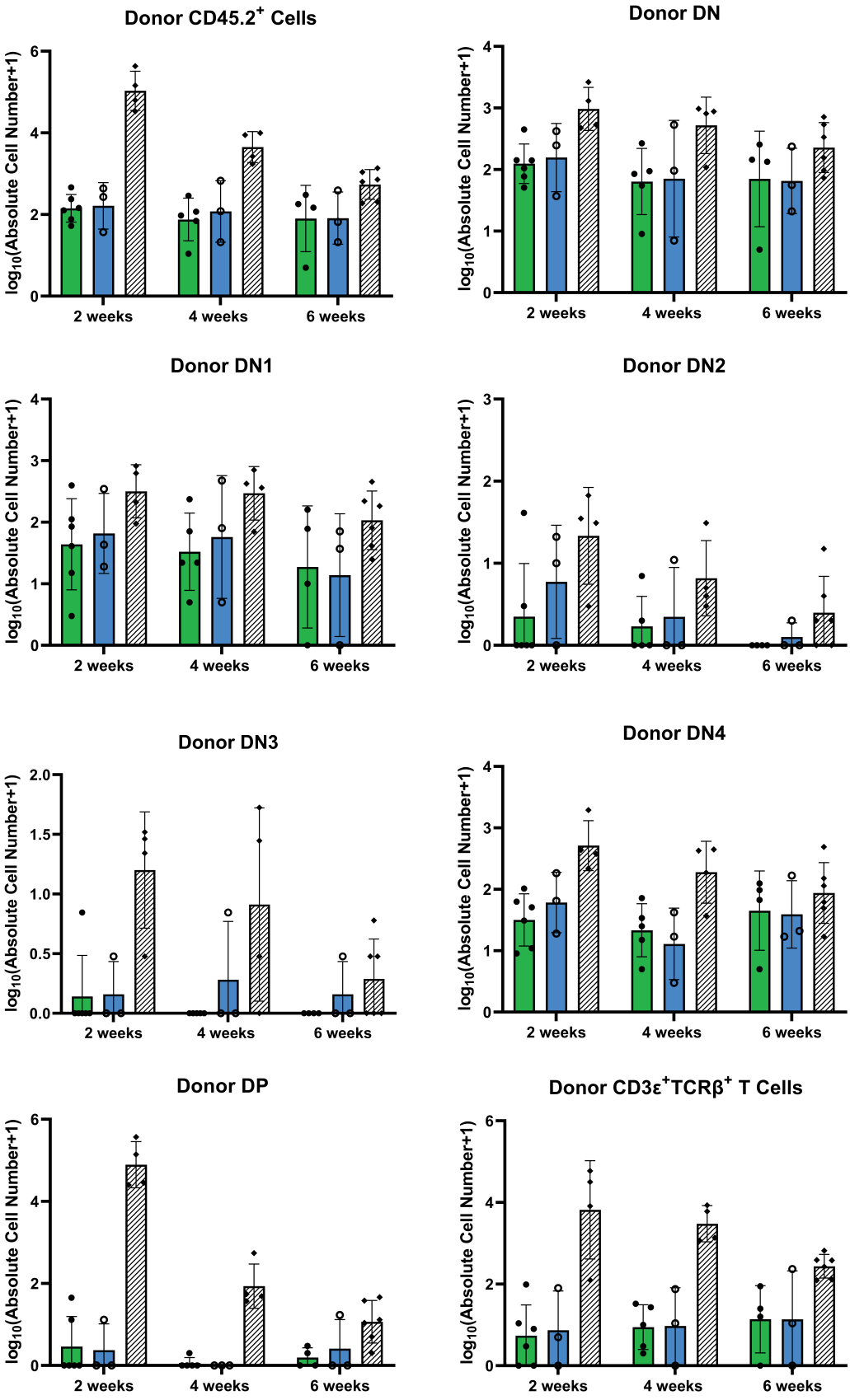
Figure 5.10. Thymopoiesis in RFTOC grafts 6 weeks post grafting. Representative FACS plots and gating hierarchy for RFTOC 6 weeks post transplantation. Transplant recipients received one RFTOC consisting of 6 dissociated whole E15.5 thymic lobes + 200,000 WT MEFs. Donor and host cells were gated from CD45.2 and CD45.1+ cells, respectively. Donor cells were obtained from WT C57BL/6J mice and grafted into host Ly5.1 homozygous C57BL/6J recipients.

Donor, t=6 weeks post-grafting															
Condition	**Animal**	**Donor Cells**	**DN**	**DN1**	**DN2**	**DN3**	**DN4**	**DP**	**SP4**	**SP8**	**CD3ε⁺TCRβ⁺**	**SP4 (CD3ε⁺TCRβ⁺)**	**SP8 (CD3ε⁺TCRβ⁺)**	**Semi-mature**	**Egressing**
iTEC MultiRTOC	1	301	255	159	0	0	96	2	41	3	24	6	0	1	2
	2	180	143	77	0	0	66	1	34	2	15	3	0	0	5
	3	147	133	9	0	0	124	0	14	0	88	8	0	1	77
	4	4	4	0	0	0	4	0	0	0	0	0	0	0	0
	Mean	**158 ± 106**	**134 ± 89**	**61 ± 64**	**0 ± 0**	**0 ± 0**	**73 ± 45**	**1 ± 1**	**22 ± 16**	**1 ± 1**	**32 ± 34**	**4 ± 3**	**0 ± 0**	**1 ± 1**	**21 ± 32**
iTEC+FTM MultiRTOC	1	389	236	70	0	0	166	16	91	46	233	72	39	4	129
	2	65	55	36	1	2	16	0	10	0	10	2	0	0	3
	3	20	20	0	0	0	20	0	0	0	0	0	0	0	0
	Mean	**158 ± 164**	**104 ± 95**	**35 ± 29**	**0 ± 0**	**1 ± 1**	**67 ± 70**	**5 ± 8**	**34 ± 41**	**15 ± 22**	**81 ± 108**	**25 ± 33**	**13 ± 18**	**1 ± 2**	**44 ± 60**
RFTOC	1	189	72	24	0	0	48	10	51	56	128	43	48	1	46
	2	634	335	182	1	2	150	4	227	68	267	78	37	13	95
	3	201	154	40	1	0	113	1	43	3	123	26	2	1	95
	4	1090	534	454	14	5	61	45	318	193	389	143	111	2	236
	5	1359	713	220	3	2	488	37	177	432	660	100	223	15	367
	6	720	96	80	0	0	16	12	164	448	380	116	240	0	356
	Mean	**699 ± 428**	**317 ± 237**	**167 ± 147**	**3 ± 5**	**2 ± 2**	**146 ± 160**	**18 ± 17**	**163 ± 96**	**200 ± 179**	**325 ± 183**	**84 ± 40**	**110 ± 92**	**5 ± 6**	**199 ± 128**

Table 5.5. Absolute cell counts of donor cell populations in recovered grafts at six weeks *in vivo*. Detailed account of absolute cell numbers obtained from each technical replicate along with mean ±SD per condition.

Host, t=6 weeks post-grafting															
Condition	**Animal**	**Host Cells**	**DN**	**DN1**	**DN2**	**DN3**	**DN4**	**DP**	**SP4**	**SP8**	**CD3ε⁺TCRβ⁺**	**SP4 (CD3ε⁺TCRβ⁺)**	**SP8 (CD3ε⁺TCRβ⁺)**	**Semi-mature**	**Egressing**
iTEC MultiRTOC	1	151826	47573	39141	660	1780	5992	92807	6548	4898	17758	3469	1751	1145	8800
	2	77727	15762	12637	349	1093	1683	57112	1966	2887	3860	788	343	103	2382
	3	4758	3405	2398	0	0	1007	117	850	386	1718	675	305	116	826
	4	1992	1524	1260	4	0	260	0	312	156	512	196	100	52	212
	Mean	**59076 ± 61562**	**17066 ± 18443**	**13859 ± 15254**	**253 ± 274**	**718 ± 758**	**2236 ± 2226**	**37509 ± 39520**	**2419 ± 2457**	**2082 ± 1947**	**5962 ± 6915**	**1282 ± 1282**	**625 ± 657**	**354 ± 457**	**3055 ± 3410**
iTEC+FTM MultiRTOC	1	349038	13146	6841	171	1745	4389	318093	12480	5319	47179	10625	2776	6994	13217
	2	33205	7532	5926	263	385	958	23487	1446	740	3102	725	205	251	1218
	3	3688	2112	1740	4	0	368	60	1168	348	1288	864	248	56	852
	Mean	**128644 ± 156308**	**7597 ± 4505**	**4836 ± 221**	**146 ± 107**	**710 ± 749**	**1905 ± 1773**	**113880 ± 144717**	**5031 ± 5268**	**2136 ± 2257**	**17190 ± 21219**	**4071 ± 4634**	**1076 ± 1202**	**2434 ± 3226**	**5096 ± 5745**
RFTOC	1	1205576	22902	8095	204	3443	11160	960249	170418	52007	286576	164315	43793	70011	40512
	2	765891	13076	2741	192	2068	8075	622000	104788	26027	118060	83140	19490	14740	74640
	3	994345	24370	7944	180	3832	12414	794175	131368	44432	199750	122769	35250	49265	29741
	4	2629171	67411	10779	1170	7930	47532	1825840	260286	475634	591677	246898	130227	39254	443239
	5	875111	58863	45337	412	1689	11425	647638	96505	72105	181700	92399	34323	20389	111823
	6	683172	13540	2612	160	2816	7952	526204	107316	36112	134244	94824	25356	8440	100824
	Mean	**1192211 ± 664021**	**33360 ± 21619**	**12918 ± 14795**	**386 ± 360**	**3630 ± 2059**	**16426 ± 14012**	**896018 ± 438294**	**145114 ± 57026**	**117720 ± 160696**	**252001 ± 161273**	**134058 ± 57218**	**48073 ± 37537**	**33683 ± 21472**	**133463 ± 141629**

Table 5.6. Absolute cell counts of host cell populations in recovered grafts at six weeks *in vivo*. Detailed account of absolute cell numbers obtained from each technical replicate along with mean ±SD per condition.


Donor CD45.2+ Cells
Donor DN
Donor DN1
Donor DN2
Donor DN3
Donor DN4
Donor DP
Donor CD3ε+TCRβ+ T Cells
log10(Absolute Cell Number+1)
2 weeks
4 weeks
6 weeks

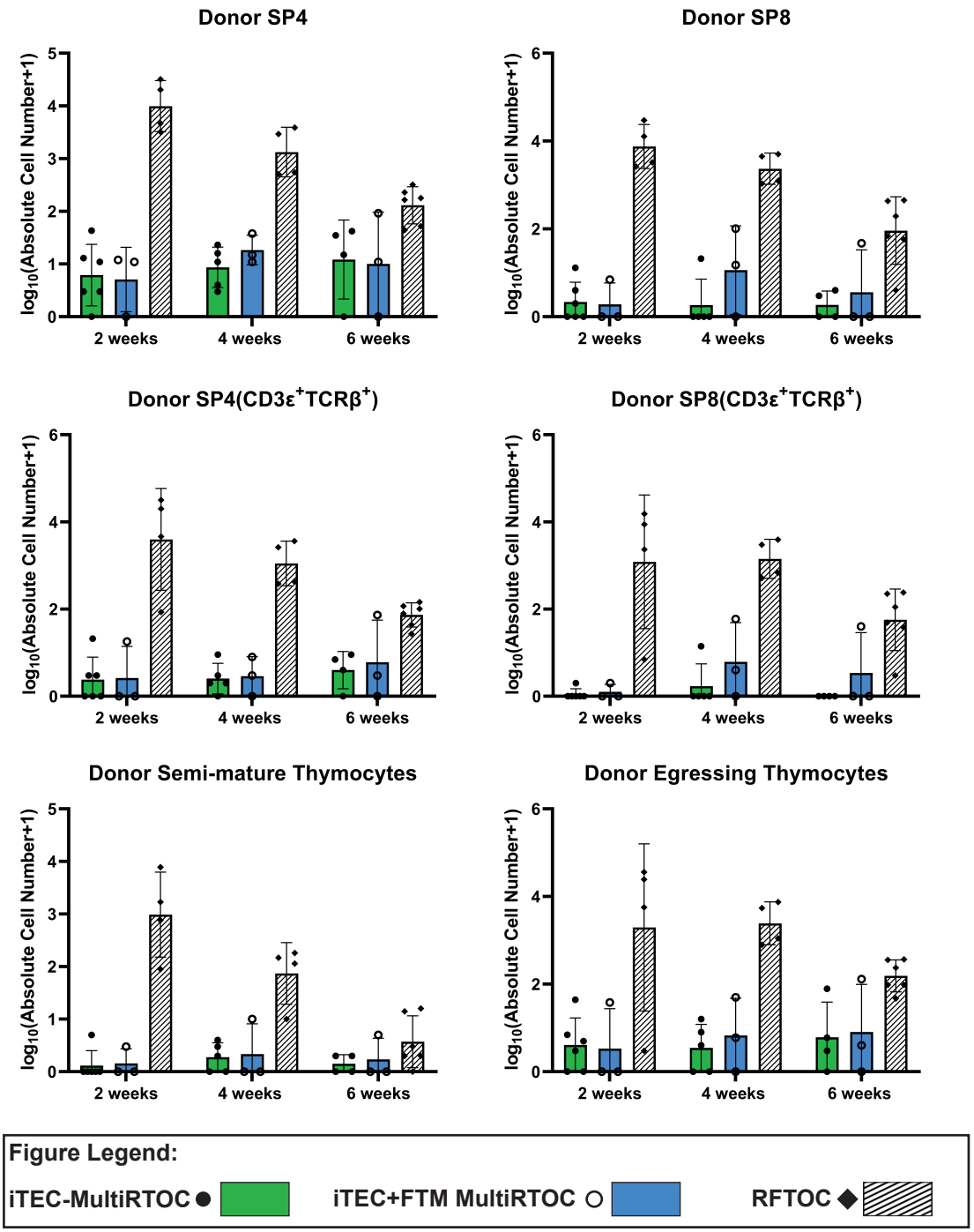
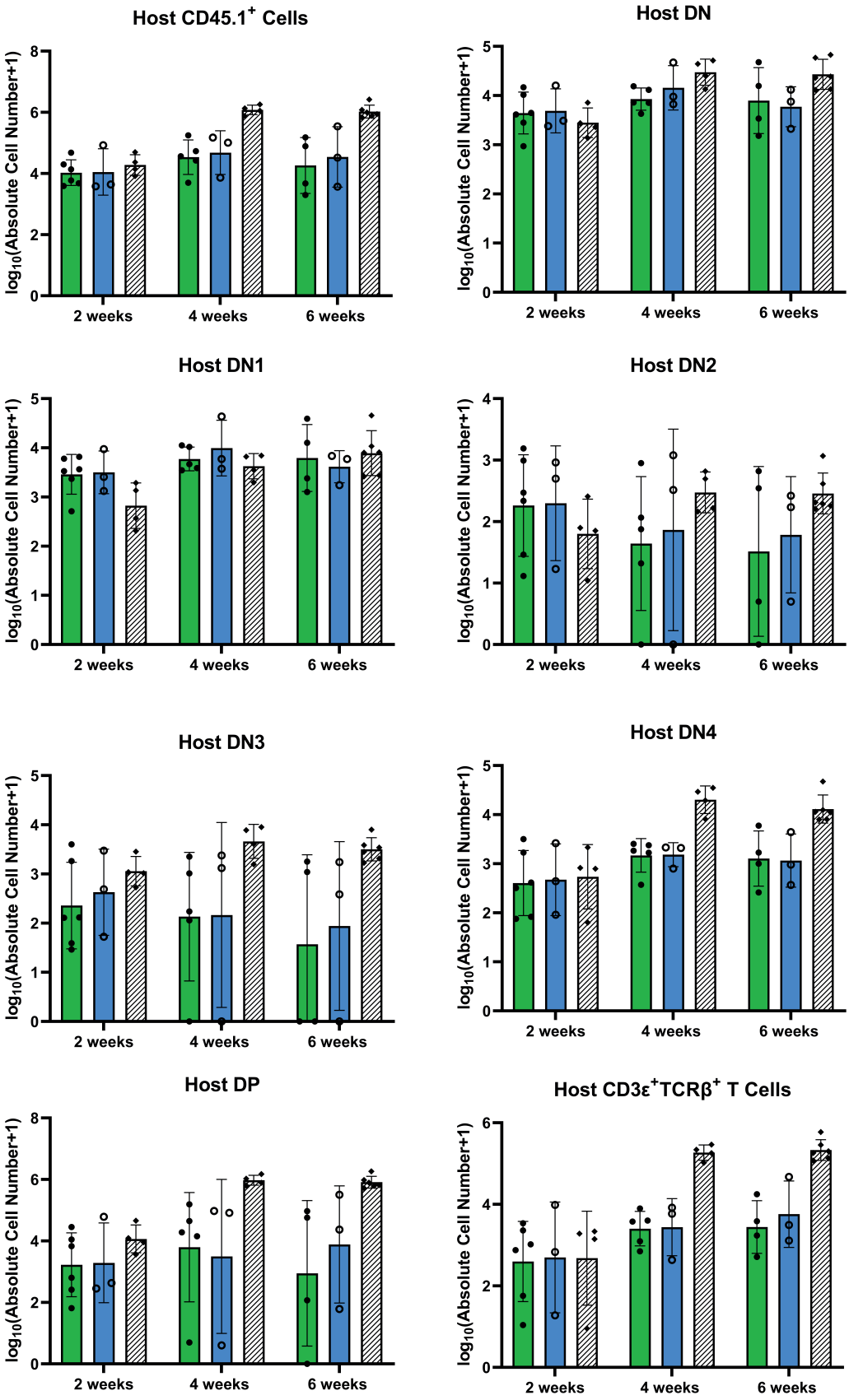


Figure 5.11. Log transformed absolute cell numbers of donor thymocytes present in grafts 2,4 or 6 weeks *in vivo* in the presence or absence of FTM in iTEC MultiRTOC. Graphs display mean+SD. DN ($CD4^-CD8^-$), DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3($CD44^-CD25^+$), DN4 ($CD44^-CD25^-$), DP ($CD4^+CD8^+$), $CD3\varepsilon^+TCR\beta^+$ T cells, SP4 ($CD4^+CD8^-$), SP8 ($CD4^-CD8^+$), Semi-mature($CD3\varepsilon^+TCR\beta^+CD69^+CD62L^-$), Egressing Thymocytes ($CD3\varepsilon^+TCR\beta^+CD69^-CD62L^+$). iTEC-MultiRTOC 3 x (600,000 iTEC + 100,000 WT MEFs + 100,000 DNs), iTEC+FTM-MultiRTOC 3 x (600,000 iTEC + 100,000 WT MEFs + 100,000 DNs + 50,000 FTM), RFTOC (6 dissociated whole E15.5 thymic lobes + 200,000 WT MEFs). Thymocytes were obtained from grafts recovered 2,4 or 6 weeks post transplantation. Each datapoint represents one animal with N=3 independent experiments in total.


Host CD45.1+ Cells
Host DN
Host DN1
Host DN2
Host DN3
Host DN4
Host DP
Host CD3ε+TCRβ+ T Cells
log10(Absolute Cell Number+1)
2 weeks
4 weeks
6 weeks

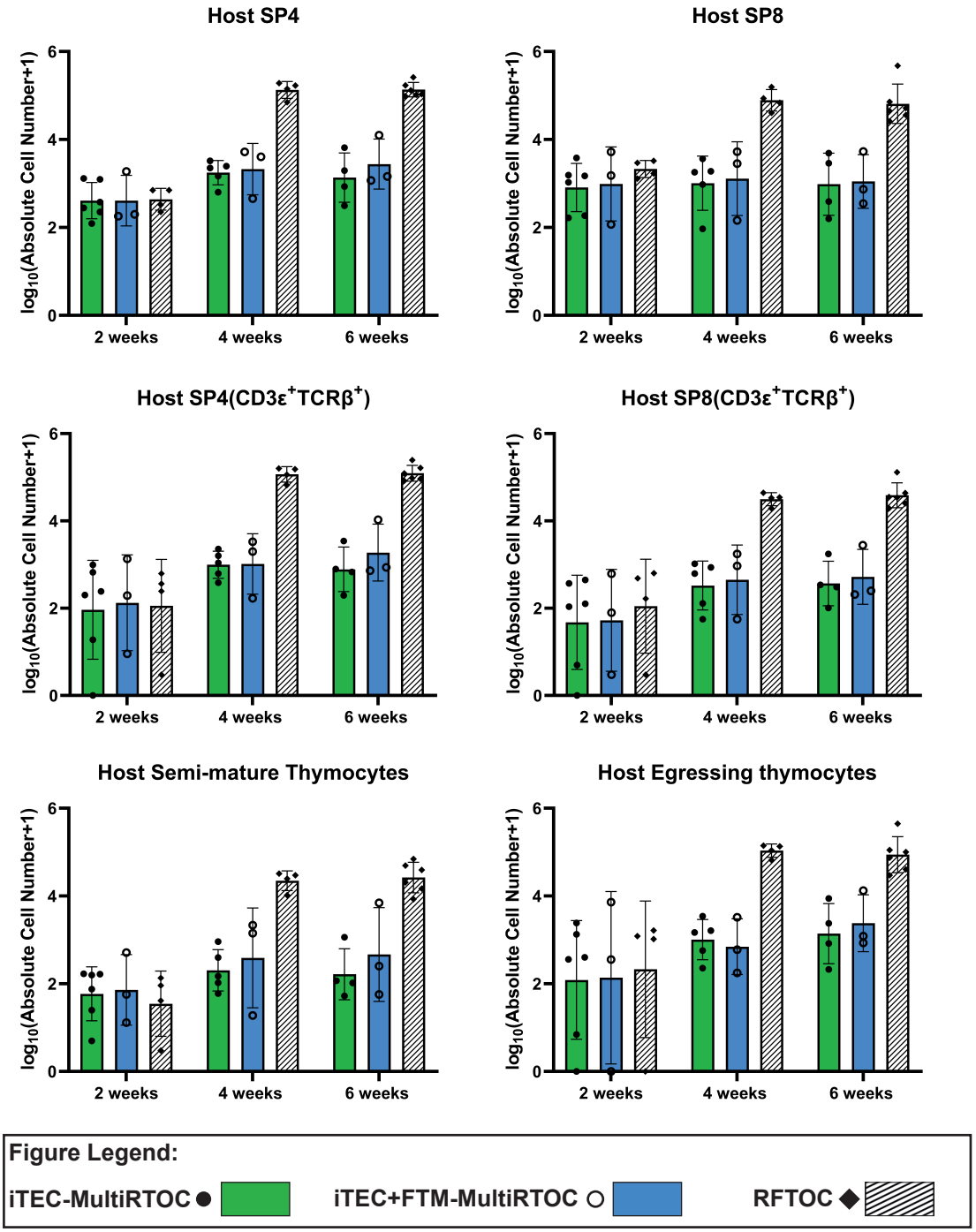


Figure 5.12. Log transformed absolute cell numbers of host thymocytes present in grafts 2,4 or 6 weeks *in vivo* in the presence or absence of FTM in iTEC MultiRTOC. Graphs display mean±SD. DN ($CD4^-CD8^-$), DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), DN4 ($CD44^-CD25^-$), DP ($CD4^+CD8^+$), $CD3\varepsilon^+TCR\beta^+$T cells, SP4 ($CD4^+CD8^-$), SP8 ($CD4^-CD8^+$), Semi-mature ($CD3\varepsilon^+TCR\beta^+CD69^+CD62L^-$),Egressing Thymocytes ($CD3\varepsilon^+TCR\beta^+CD69^-CD62L^+$). iTEC-MultiRTOC 3 x (600,000 iTEC + 100,000 WT MEFs + 100,000 DNs), iTEC+FTM-MultiRTOC 3 x (600,000 iTEC + 100,000 WT MEFs + 100,000 DNs + 50,000 FTM), RFTOC (6 dissociated whole E15.5 thymic lobes + 200,000 WT MEFs). Thymocytes were obtained from grafts recovered 2,4 or 6 weeks post transplantation. Each datapoint represents one animal with N=3 independent experiments in total.

Condition		2 weeks post-grafting			4 weeks post-grafting			6 weeks post-grafting		
		iTEC MultiRTOC	iTEC+FTM MultiRTOC	RFTOC	iTEC MultiRTOC	iTEC+FTM MultiRTOC	RFTOC	iTEC MultiRTOC	iTEC+FTM MultiRTOC	RFTOC
Donor	Donor Cells	183	247	168954	116	269	5794	158	158	699
	DN	160	234	1238	101	211	692	134	104	317
	DN1	108	135	435	70	186	390	61	35	167
	DN2	7	10	33	1	3	10	0	0	3
	DN3	1	1	21	0	2	20	0	1	2
	DN4	44	88	746	29	20	272	73	67	146
	DP	9	4	140611	0	0	171	1	5	18
	SP4	11	7	15029	10	20	1968	22	34	163
	SP8	3	2	12076	4	38	2963	1	15	200
	$CD3\varepsilon^{+}TCR\beta^{+}$	19	28	24837	14	28	4287	32	81	325
	$SP4(CD3\varepsilon^{+}TCR\beta^{+})$	4	6	14092	2	3	1768	4	25	84
	$SP8(CD3\varepsilon^{+}TCR\beta^{+})$	0	0	6617	3	20	2028	0	13	110
	Semi-mature	1	1	2576	1	3	112	1	1	5
	Egressing	9	12	16561	5	18	3700	21	44	199

Table 5.7. Mean absolute cell counts of donor cell populations in recovered grafts at two, four or six weeks *in vivo*. Mean absolute cell count per condition at each timepoint.

Condition		2 weeks post-grafting			4 weeks post-grafting			6 weeks post-grafting		
		iTEC MultiRTOC	iTEC+FTM MultiRTOC	RFTOC	iTEC MultiRTOC	iTEC+FTM MultiRTOC	RFTOC	iTEC MultiRTOC	iTEC+FTM MultiRTOC	RFTOC
Host	**Host Cells**	16083	30656	23768	59961	86000	1261573	59076	128644	1192211
	DN	6242	7128	3395	9431	21110	33798	17066	7597	33360
	DN1	3745	4427	965	6735	17690	4773	13859	4836	12918
	DN2	514	477	104	220	509	366	253	146	386
	DN3	1027	1171	1373	714	1234	5599	718	710	3630
	DN4	956	1052	954	1762	1677	23060	2236	1905	16426
	DP	7875	20494	17586	46838	58892	997324	37509	113880	896018
	SP4	589	753	490	2017	3248	142842	2419	5031	145114
	SP8	1377	2282	2298	1675	2751	87609	2082	2136	117720
	CD3ε$^+$TCRβ$^+$	1264	3441	1373	3517	4848	198774	5962	17190	252001
	SP4(CD3ε$^+$TCRβ$^+$)	352	517	307	1195	1824	123625	1282	4071	134058
	SP8(CD3ε$^+$TCRβ$^+$)	175	232	321	540	911	32891	625	1076	48073
	Semi-mature	99	193	67	323	1200	24264	354	2434	33683
	Egressing	753	2539	975	1472	1344	112680	3055	5096	133463

Table 5.8. Mean absolute cell counts of host cell populations in recovered grafts at two, four or six weeks *in vivo*. Mean absolute cell count per condition at each timepoint.

5.2.B. iTEC recruit host lymphohaematopoietic progenitor cells (LPCs) and support their T cell lineage commitment and development

Analysis of the recovered iTEC grafts revealed that very few donor T cells were present by two weeks post-grafting. On average, there were less than 185 donor cells still present, with the majority being DN1. The same pattern was observed at all three timepoints analysed. These results do not indicate whether the majority of the donor haematopoietic cells died following transplantation, or if they underwent thymopoiesis and entered the peripheral immune system. The positive controls contained large cell numbers of donor T cells at two weeks, with drastically decreased numbers by four weeks and almost no donor cells remaining by six weeks (29-fold decrease in two weeks).

Analysis of the presence of host haematopoietic cells revealed that, by two weeks post-grafting, large numbers of host haematopoietic cells were present in iTEC grafts and these numbers increased over time. On average, 16,083 cells were present by two weeks (38% DN, 48% DP) in iTEC-MultiRTOC, whereas 30,656 (23% DN, 67% DP) were present in iTEC+FTM-MultiRTOC. By four weeks, iTEC-MultiRTOC contained 59,961 host cells (15% DN, 78% DP), whereas iTEC+FTM-MultiRTOC had 86,000 cells present (24% DN, 68% DP). By six weeks, iTEC-MultiRTOC contained 59,000 cells (28% DN, 62% DP), while iTEC+FTM-MultiRTOC contained 128,644 cells (6% DN, 88% DP). For all conditions, except iTEC-MultiRTOC at six weeks, the number of host cells present in the graft increased over time.

This pattern was also followed by a proportional reduction in DN and a proportional increase in DP stage cells. Taken in combination, the fact that the numbers of both DN and DP increase over time suggests continuous recruitment of LPCs to replenish cells that have progressed from DN to DP, or profound proliferation of DNs is taking place.

Inclusion of FTM in the MultiRTOC system at all three timepoints resulted in increased numbers of host cells with 1.9-, 1.4- and 2.2-fold increases in total host cells present compared to the corresponding iTEC-MultiRTOC conditions at two, four and six weeks, respectively. However, due to the limitation in numbers of FTM cells that could be harvested for each experiment, only one animal was grafted with iTEC+FTM-MultiRTOC per experiment (three in total/condition). For iTEC-MultiRTOC, a higher number of animals per timepoint was analysed, allowing for

more variation to be captured. For example, for the iTEC+FTM-MultiRTOC condition, animal 2 at two weeks contained a high number of host cells, increasing the average for total host cells, DN and DP T cells. At four weeks, two of three animals exhibited high host cell numbers with the third one containing far fewer. At six weeks animal 1 outperformed the others, again increasing the average. Due to the low number of animals examined for the iTEC+FTM-MultiRTOC condition, clear conclusions were not able to be made on the effect of FTM in iTEC-MultiRTOC. This requires repetition but was not within the scope of this PhD due to limited animals available.

All conditions displayed an increase in cell number over time in all SP4 and SP8, subsets examined: $CD3\varepsilon^{+}TCR\beta^{+}$ T cells, SP4 ($CD3\varepsilon^{+}TCR\beta^{+}$), SP8 ($CD3\varepsilon^{+}TCR\beta^{+}$), semi-mature ($CD69^{+}CD62L^{-}$) and egressing ($CD69^{-}CD62L^{-}$) T cells suggesting that the recruited host LPCs completed normal thymopoiesis.

The numbers of T cells present in iTEC-MultiRTOC and positive control RFTOC were very similar at two weeks *in vivo*, however, appear vastly increased by the four-week timepoint with total host cells present being above 10^{6} cells. The fact that iTEC and RFTOC conditions resemble similar cell numbers at 2 weeks but differ greatly after this suggests that iTEC may not display as good a recruitment efficiency as native TEC, thymocytes present do not proliferate as much in iTEC conditions, there is a T cell blockade at some stage of differentiation or iTEC do not proliferate and offer an expanding niche to support an increasing number of thymocytes.

5.2.C. Do grafted iTEC-RTOC require donor haematopoietic progenitor cells?

To examine whether donor LPCs are a requirement of the grafted iTEC-RTOC system, I designed an experiment in which multiple RTOC were transplanted, that consisted of only iTEC and WT MEFs (Figure 5.13.A). For the iTEC condition three RTOC containing 200,000 iTEC + 100,000 WT MEFs were transplanted and their ability to support recruited host LPCs was examined along with the histology of recovered grafts. Negative control recipients received the same cell number, with iTEC being replaced by Cre MEFs. Grafts were left for four weeks before analysis.

Two completely independent biological replicates were performed for the flow cytometry analysis, with two animals transplanted per experiment, and one biological replicate was performed for negative control results, due to lack of mice available for transplantation. Data points were kept separate to capture variability within the same experiment.

For the histological analyses, the experiment was only carried out once, two animals were transplanted with the same iTEC condition and compared. This experiment was carried out prior to the analyses described in section 5.B above and provided the first indication that iTEC grafts can recruit host LPCs and support their development into T cells.

The iTEC-only reaggregate transplants were recovered four weeks after transplantation. The recovered grafts contained $CD45^+$ haematopoietic cells. A high percentage of the $CD45^+$ haematopoietic cells present were in the DP stage of T cell development (Figure 5.13.B).

The presence of $CD45^+$ cells and their presence in the DP stage of T cell development indicates that recruitment of host LPCs occurs in iTEC-only grafts, and that these become committed to the T cell lineage and are supported through to the DP stage of thymopoiesis. Flow cytometry analysis was performed after staining for CD45, CD4, CD8, CD3ε, TCRβ, FOXP3 and Fixable Viability Dye (FVD) eFluor450. Percentages of parent gate were calculated for $CD45^+$ live cells, DN ($CD4^-CD8^-$), DP ($CD4^+CD8^+$), SP4 ($CD4^+CD8^-$), SP8 ($CD4^-CD8^+$), T cells ($CD3\varepsilon^+TCR\beta^+$), and Tregs ($CD4^+FOXP3^+$) (Table 5.9). Representative FACS plots and gating hierarchy can be seen in Figure 5.14 (Cre) and 5.15 (iTEC).

The Cre negative control graft did not contain any DP cells even though $CD45^+$ cells were present, so these are likely to represent recirculating T cells from the host (SP4, SP8 and $CD3\varepsilon^+TCR\beta^+$ T cells) as well as cells of other haematopoietic lineages. The iTEC graft recipients displayed varying levels of haematopoietic cells as a proportion of the total graft cellularity, varying from 1.9% through to 10.12%. All the iTEC grafts contained DP T cells present, indicating active thymopoiesis. These data establish that iTEC grafts generated without donor thymocytes or fetal thymic mesenchyme can attract host LPCs and support their commitment to and development along the T cell lineage. However, the size of the recovered grafts suggested that these grafts were not as robust as iTEC-MultiRTOC that contained donor thymocytes.

H&E analysis of the recovered iTEC grafts revealed regions of hematoxylin staining cell nuclei characteristic of T cells in the thymus (Figure 5.16). They also contained large regions of eosin staining indicating an absence of T cells. Overall, the recovered grafts lacked classical thymus morphology. Recovered grafts presented in Figure 5.16 are 0.8 mm x 0.35 mm and 1.2 mm x 0.56 mm (Width x Height) respectively. For comparison, the same condition but containing donor thymocytes is presented in Chapter 4 in Figure 4.1.12 and RepA which resembles native thymic architecture is 1.5 mm x 0.72 mm. Immunohistochemical staining with αK8, UEA1 and αCD45 revealed the presence of all three markers within the grafts, but no organisation into distinct cortical and medullary regions was evident (Figure 5.16).

When compared to the histology of recovered grafts of iTEC-RTOC that contained donor thymocytes (shown in Chapters 3 and 4), it is evident that the histology of recovered grafts of iTEC+MEFs-only reaggregates show fewer characteristics of the native thymus. This suggests that donor thymocyte presence and therefore thymic crosstalk, possibly early after iTEC-RTOC formation, may have a positive impact on the capacity of iTEC to organise into cortical and medullary compartments and to attain their full functionality. As previously shown, the organisation of iTEC grafts into cortical and medullary regions occurs only in a proportion of grafts. Therefore, these data highlight the importance of donor lymphocytes in establishing graft organisation. As the availability of donor thymocytes was not an issue and these experiments are very time-consuming and use animals, it was decided going forward to leave donor thymocytes in the iTEC-RTOC system.

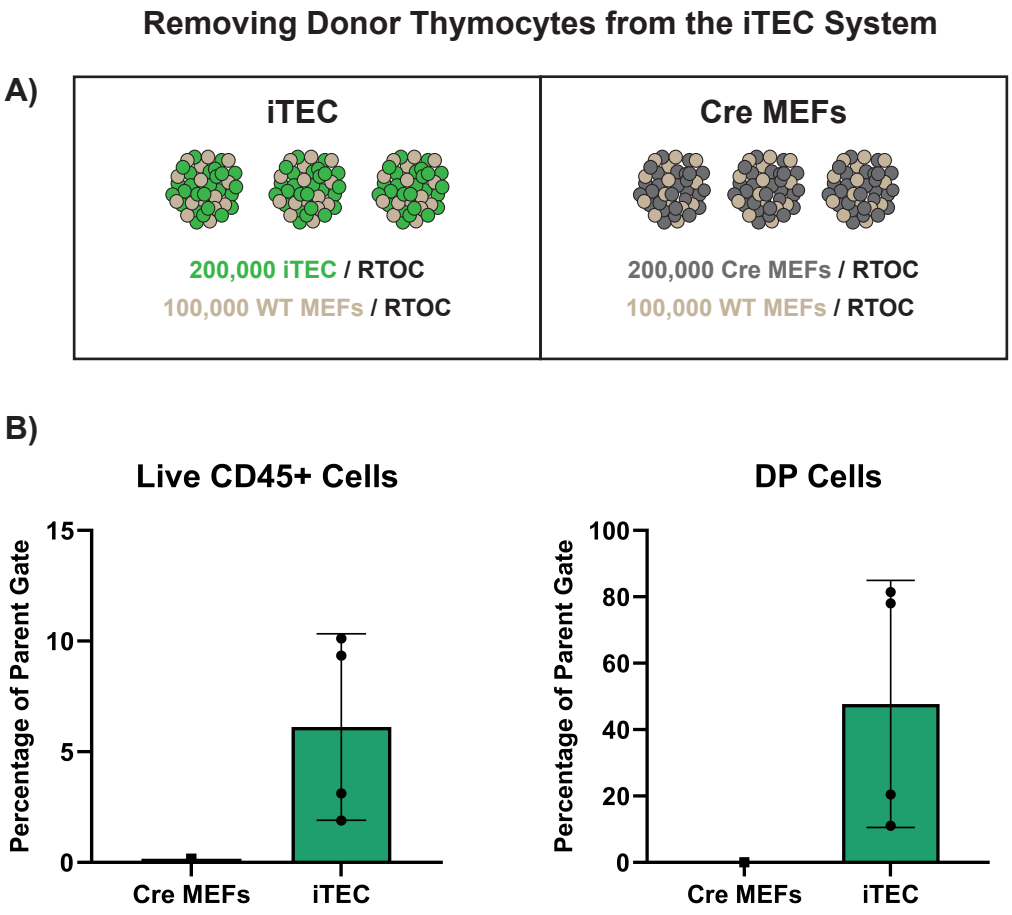


Figure 5.13. Transplantation of iTEC with WT MEFs is capable of recruiting host lymphoid progenitors and supporting thymopoiesis. All cells are syngeneic to the host (WT C57BL/6J). **A)** For the iTEC condition three RTOC consisting of 200,000 iTEC and 100,000 WT MEFs each were transplanted under the kidney capsule. For the Cre MEFs condition three RTOC consisting of 200,000 Cre MEFs and 100,000 WT MEFs each were transplanted under the kidney capsule. **B)** Barplots of Live $CD45^+$ and DP ($CD45^+CD4^+CD8^+$) cells present within the grafts 4 weeks post-transplantation. Each datapoint represents one animal. N=2 (2 animals/ experiment) independent experiments for iTEC and N=1 experiment for Cre MEFs.

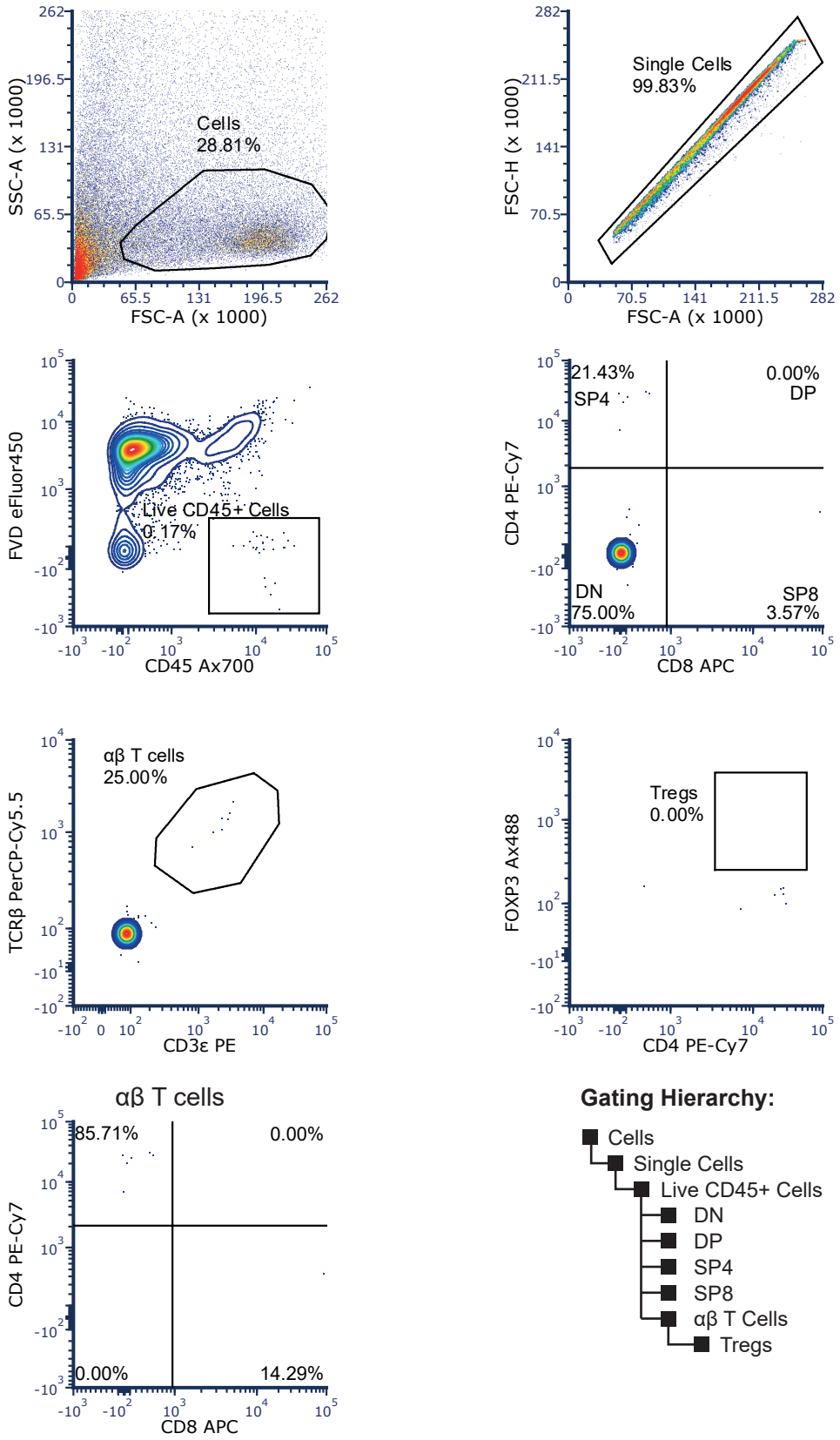


Figure 5.14. RTOC consisting of Cre MEFs and WT MEF are not able to recruit lymphoid progenitors and support T Cell lineage commitment and development. Representative FACS plots 4 weeks *in vivo*. Transplant recipients received three RTOC consisting of 200,000 Cre MEFs +100,000 WT MEFs.

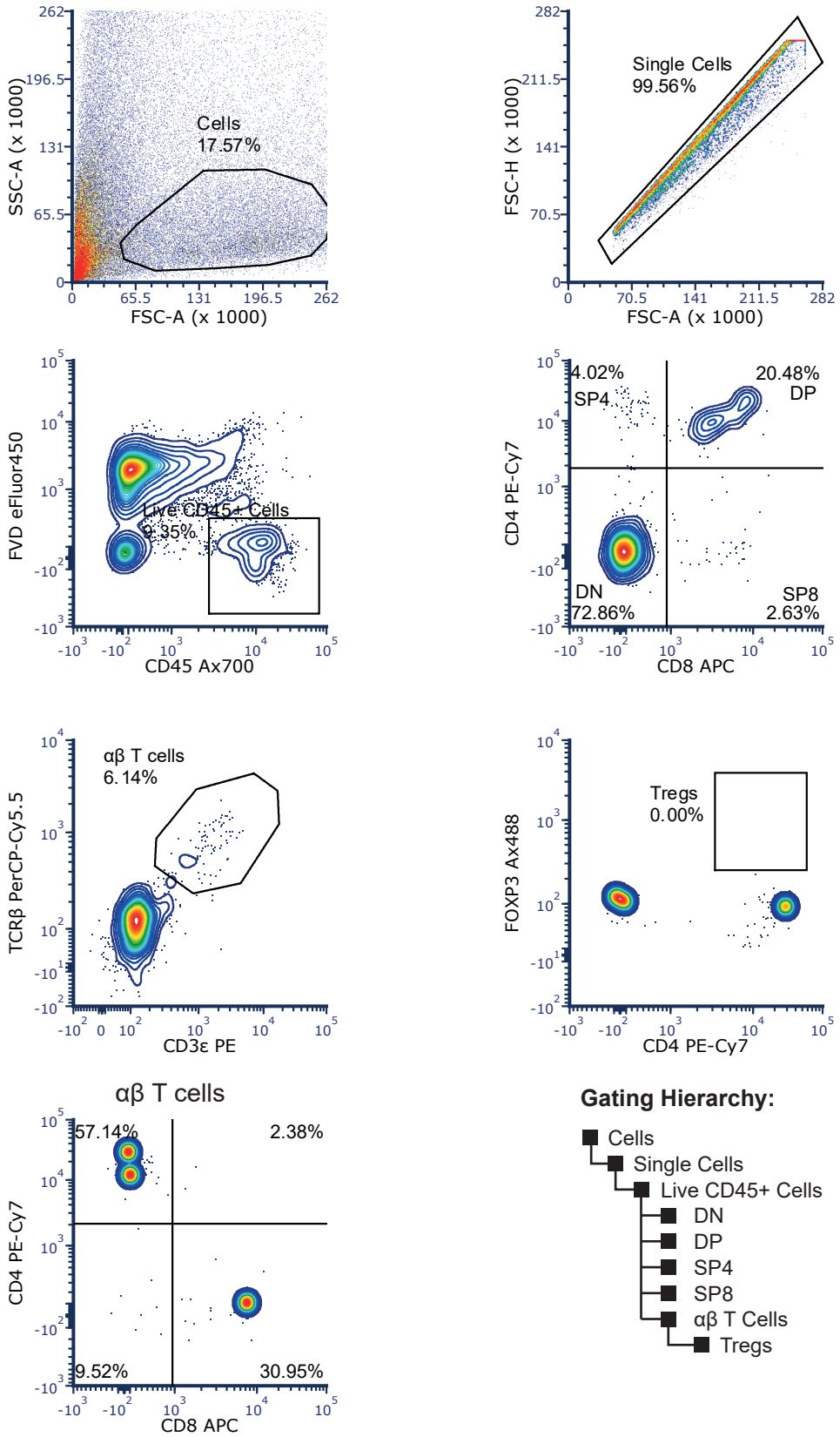


Figure 5.15. RTOC consisting of iTEC and WT MEF are able to recruit lymphoid progenitors and support T Cell lineage commitment and development.
Representative FACS plots 4 weeks *in vivo*. Transplant recipients received three RTOC consisting of 200,000 iTEC +100,000 WT MEFs.

Condition	Animal	CD45$^+$	DN	DP	SP4	SP8	CD3ε$^+$TCRβ$^+$	Tregs
Cre MEFs	1 (1st EXP)	0.17	75	0	21.43	3.57	25	0
iTEC + MEFs	1 (1st EXP)	10.12	16.5	78.07	3.98	1.45	5.39	0.77
	2 (1st EXP)	3.12	11.02	81.43	5.1	2.45	5.1	0
	3 (2nd EXP)	1.9	68.9	11	12.2	7.89	20.1	0
	4 (2nd EXP)	9.35	72.86	20.48	4.02	2.63	6.14	0
	Mean	**6.12 ± 3.64**	**42.32 ± 28.65**	**47.75 ± 32.20**	**6.33 ± 3.42**	**3.61 ± 2.51**	**9.18 ± 6.31**	**0.19 ± 0.33**

Table 5.9. Cell percentages of parent gate for the cell populations shown, in recovered grafts transplanted with iTEC or Cre MEFs plus WT MEFs, recovered 4 weeks post-transplantation. Cell percentages of each animal along with mean ±SD for iTEC condition.

iTEC and WT MEF only MultiRTOC
3 X RTOC (200,000 iTEC + 100,000 WT MEFs)

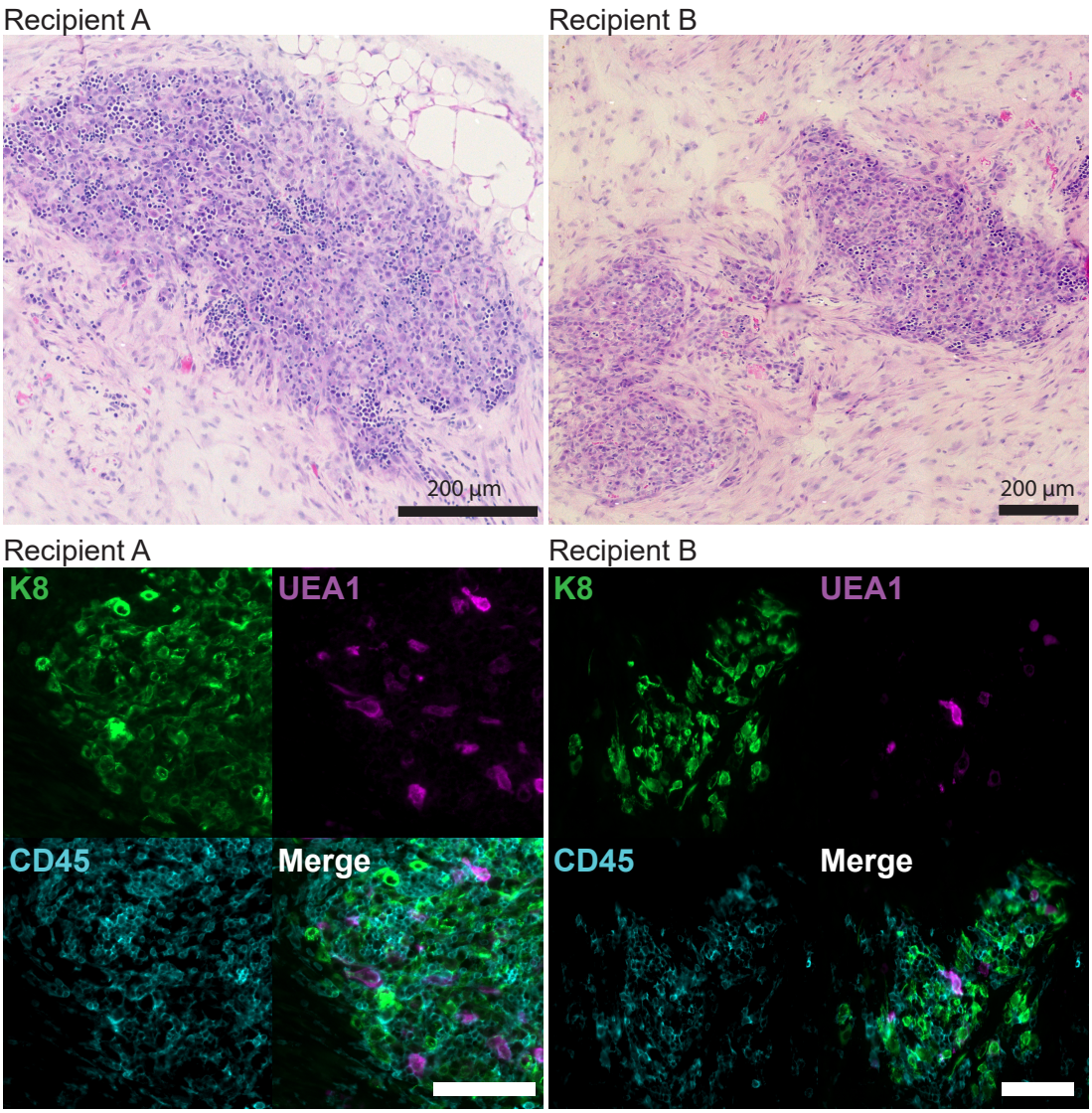


Figure 5.16. Transplantation of iTEC with WT MEFs is capable of recruiting host haematopoietic cells. Histology of recovered iTEC-MultiRTOC grafts 3 x (200,000 iTEC + 100,000 WT MEFs). H&E (top panel), IHC (bottom panel). Grafts were stained with cytokeratin 8-K8 (green) for cortical regions, UEA1 (magenta) for medullary regions and CD45 (cyan) for haematopoietic cells. 100 µm scale bar for IHC images. Data shown was carried out for N=1 experiment. Left is iTEC recipient A and right is iTEC recipient B. Graft recovered for Cre MEFs recipient was not large enough to histologically examine.

5.3. Discussion

This Chapter focuses on contribution of donor versus host iTEC-derived T cells within grafts. I have also examined the complete removal of other cell types (bar MEFs) from iTEC-RTOC. An alternative approach for iTEC transplantation was explored with the administration of increased amount of RTOC and iTEC numbers per recipient to compensate for the lack of FTM, which is the biggest limiting factor of these experiments. The ability of iTEC to recruit host lymphohaematopoietic progenitors and support their T cell lineage commitment and development was determined. Finally, the importance of donor DN thymocytes in iTEC-MultiRTOC that contain only iTEC and WT MEFs was explored.

5.3.A. LPC Recruitment

In section 5.2.B of this chapter, the ability of iTEC to recruit host lymphoid progenitors (LPCs) and support their development was explored. The thymus does not contain any HSCs with long-term self-renewal capacity, or indeed any other self-renewing haematopoietic population, and therefore requires recruitment of BM-derived progenitors from the circulation to support T cell generation throughout the lifespan (Scollay, Smith and Stauffer, 1986; Donskoy and Goldschneider, 1992; Zlotoff and Bhandoola, 2011).

HSCs in the bone marrow give rise to multipotent progenitors (MPPs) which then become lymphoid-primed MPPs (LMPPs), collectively known as LSK ($Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$). LMPPs give rise to common LPCs (Zlotoff and Bhandoola, 2011). Rare numbers of LPCs enter the thymus, described as thymic settling progenitors, and undergo thymopoiesis. Only a handful of lymphoid progenitors enter the thymus each day and once entered these cells are thought to persist for only several weeks (Scollay, Smith and Stauffer, 1986). It has been shown postnatally that 2-3% of LPCs are replaced daily (Donskoy and Goldschneider, 1992).

Thymopoiesis is characterised by several proliferative thymocyte bursts taking place at specific checkpoints. Early thymic progenitors (ETPs) undergo 1,000-fold expansion upon entry to the thymus over the course of ten days (Shortman *et al*., 1990; Petrie, Carlos and Niga-Pfï Ucker, 2007). More recently it has been shown that membrane bound KitL(on vascular endothelial cells)-c-Kit(on ETPs) interactions coordinate vascular endothelial cell and ETP expansion (Buono *et al*., 2018), and highlights the role of vascularisation in this process. Interestingly so, it has been shown that robust proliferation of ETPs, and possibly downstream thymocytes, can

compensate for LPC entry defects in animal models such as CCR7(-/-) CCR9(-/-) double knockout mice which display normal thymic cellularity despite the reduced number of ETPs (Krueger *et al.,* 2010; Vicente *et al*., 2010; Zlotoff *et al*., 2010). When receiving Notch signalling at the DN1 stage for T cell lineage commitment, thymocytes upregulate CD25 and transition to the DN2 phase and undergo a further wave of proliferative expansion (Penit, Vasseur and Papiernik, 1988). Thymocytes that successfully pass β-selection transition into the DN4 stage and undergo further proliferation (Petrie *et al*., 1995; Petrie, Carlos and Niga-Pfi Ucker, 2007). Lastly SP thymocytes also undergo one last expansion in a clonotypic manner, prior to emigration to the periphery, with this mediated by TCR signalling, based on the higher range of the low affinity range for self-peptide:MHC ligands that have passed negative selection (Pénit and Vasseur, 1997; le Campion, Vasseur and Pénit, 2000; Léaument *et al*., 2002).

The experiments described in this Chapter investigated the presence of developing host thymocytes at all major stages of T cell development from DN through to SP. These demonstrated a numerical increase in all subsets assayed over time, from two to four to six weeks post-transplantation, suggesting that either: (i) recruitment takes place more than once and the starting population of LPCs entering the iTEC grafts is replenished or (ii) certain T cell subsets are undergoing robust proliferation bursts. There is the possibility that the increase in T cells over time could be due to proliferation bursts following major steps of thymopoiesis such as those occurring after colonisation, commitment, β-selection and SP4/SP8 fate decision.

In these experiments, donor thymocytes were completely depleted in iTEC-MultiRTOC by the two-week time point or remained as DN stage cells. It is not known whether the remaining donor thymocytes completed successful thymopoiesis and entered the peripheral immune system or underwent apoptosis in the RTOC. An experiment that examined the origin of the T cell populations found in the blood, lymph nodes and spleen of the grafted animals could be used to determine this. The latter would be crucial in order to understand whether acceptance of differentiated donor T cells takes place in the host and what the distribution of these subsets looks like. Although grafts with FTM in some case resulted in a higher overall number of host thymocytes, due to high variability between experiments it was not determined whether FTM had an impact on LPC recruitment or just improved early T cell development as discussed previously.

5.3.B. Investigation into the requirement of donor thymocytes for iTEC-RTOC transplantation

Transplantation of allogeneic donor BM derived cells or peripheral blood stem cells can result in graft vs host disease (GvHD), whereby transplanted T cells in the graft elicit an immune response again the host (Shlomchik, 2007). It is therefore highly advantageous for a system such as iTEC-RTOC, that aims to treat T cell immunodeficiencies, that it does not rely on supply of donor LPCs that may lead to immunological complications but rather utilises the hosts own LPCs. In the future the iTEC system could be utilised to be patient specific so that they are selecting for T cells with the same set of major and minor MHC molecules as the host. In the case where iTEC are not fully syngeneic to the host it would be expected that positive selection would take place partly on the wrong haplotype resulting in host-reactive clones. In addition, negative selection would also be partly on the wrong haplotype and set of minors. Interestingly so, Markert, Devlin and McCarthy (2010) report that HLA-mismatched donor thymocytes were positively selected for and underwent successful thymopoiesis and protected the host from infection, in DGS patients that had been transplanted with allogeneic thymic tissue. They also report the possibility that host DCs infiltrating the graft may be the ones responsible for mediating negative selection to overcome HLA-mismatch issues. However, the mechanisms behind positive and negative selection in allogeneic thymic tissue transplantation are not characterised but theories surround contribution of host cells to positive selection in allografts: (i) recipient thymocytes presenting self MHC to each other, (ii) epithelial cells recruited to allograft and (iii) host DCs in the cortex (Markert, Devlin, McCarthy, 2010). As this system is ill defined it would be beneficial to keep it as simple as possible and eliminate the need for donor thymocytes that may lead to immunological complications.

The ‘iTEC-only’ condition represents a system that is not limited by donor tissue availability and relies solely on reprogrammed iTEC plus WT MEFs, that can be cultured in abundance, and would therefore be the easiest to scale up for future clinical use. As both iTEC and WT MEFs can be generated in abundance this would offer a good system for meeting T cell immunodeficiency patient tissue needs for thymus transplantation.

It was found that iTEC-MultiRTOC in the absence of donor thymocytes can support thymopoiesis of recruited LPCs but that the histology of recovered grafts four-weeks

post transplantation was not as similar to native thymus (abundance of thymocytes, distinct cortical and medullary regions) as when donor thymocytes were present. These histology results were based on two grafted animals. As seen in Chapter 3 and Chapter 4, the histology of recovered grafts within the same batch of experiments and conditions varied considerably. So, although the histology appeared not optimal, we cannot yet rule out that this condition has the ability to produce a structure similar to that of the native thymus. However, the hypothesis that iTEC-MultiRTOC consisting solely of iTEC and WT MEFs, in the absence of DNs, are sufficient to direct host T cell development was confirmed.

The reason behind the improved histology of iTEC-MultiRTOC remains unknown but one contributing factor could be thymic crosstalk. Studies have shown that reciprocal interactions between developing thymocytes and thymic epithelial cells, named thymic crosstalk, is important for the maturation and expansion of TEC. Thymic regeneration, cTEC differentiation, medullary differentiation and organisation alongside self-tolerance induction have all been linked to thymic crosstalk (Klug *et al.*, 1998; Dudakov *et al*., 2012; Williams *et al*., 2014; Kaneko *et al*., 2019; Nitta *et al*., 2020).

Van Ewijk and colleagues first described the term ‘thymic crosstalk’ after finding that development of cortical and (particularly) medullary architecture was reliant on interactions with developing thymocytes (van Ewijk, Shores and Singer, 1994). Van Ewijk later went on to describe the importance of thymic crosstalk in shaping the architecture of these intrathymic niches and specifically the cortical and medullary environments required to support T cell development (van Ewijk *et al*., 1999, 2000). Some of the studies carried out in order to elucidate thymic crosstalk are discussed in detail in Chapter 1 and focus specifically on the requirement of thymic crosstalk in medullary expansion and cortical architecture. It was reported that early TEC development did not require TEC-thymocyte crosstalk to occur in order for TEC progenitors to differentiate and form fully functional and phenotypically correct cortical and medullary regions (Jenkinson *et al*., 2005). The data presented in that study, compares E15.5 WT lobes to E15.5 CD3εtg26 (block at DN1 stage) lobes prior and after 5 days of culture in 2-dGuo (depletes T cells). The group reports the presence of all $K5^{+}K8^{+}$ (TEPC), $K5^{-}K8^{+}$ (cTEC) and $K5^{+}K8^{-}$ (mTEC) TEC subsets in both WT and CD3εtg26 E15.5 thymic lobes at similar proportions upon collection and maintenance of $K5^{+}$ and $K8^{+}$ cells following 5 days treatment with 2-dGuo. In

both conditions following culture in 2-dGuo the TEC expressed similar levels of *gklf* (cTEC)*, plunc* and *aire* (mTEC) and were able to support DN to DP and DP to SP T cell development of WT thymocytes introduced into organ cultures with similar proportions in both conditions. So more specifically their results show that TEC development is thymocyte independent up until E15.5 of embryonic development.

Although Jenkinson *et al.* reported similar proportions of TEPC ($K5^+K8^+$) and cTEC ($K8^+K5^-$) in E15.5 WT and CD3εtg26 thymic lobes, Klug and colleagues (1998) report that this is not the case in the adult thymus. Following subrenal capsule transplantation of CD3εtg26 newborn thymi (DN1 block) into *Rag1*$^{-/-}$ mice (DN3 block), cTEC organisation and structure (reported to lack typical radial organisation in CD3εtg26) is restored demonstrating the importance of thymic crosstalk during early stages of T cell development on the cortex (Klug *et al*., 1998). In contrast when newborn CD3εtg26 thymi are transplanted into CD3εtg26 for four weeks the recovered grafts only presented with $K5^+K8^+$ cells (Klug *et al*., 1998). *Rag1*$^{-/-}$ mice thymi despite containing both TEPC and cTEC populations with normal organisation, but no medullary region formation, have a higher proportion of TEPCs than WT thymi suggesting a role of DN thymocytes in cTEC development or expansion and later stage thymocytes in medullary expansion (Klug *et al*., 1998). Klug and colleagues later published a follow up study whereby they show that during early fetal development TEC develop independently of thymocyte crosstalk but that these signals are required during late fetal development for normal neonatal and adult thymic epithelial compartment patterning (Klug *et al*., 2002). The group confirmed this through immunohistological analysis of K8 and K5 staining patterns in RAG2/γc-deficient$^{-/-}$ and Ikaros$^{-/-}$ thymi at E13.5, E15.5 that thymic crosstalk was not required at these stages for TEC development.

In order to determine if the improved histology of iTEC grafts containing donor thymocytes, in comparison to those lacking, is due to thymic crosstalk benefiting iTEC maturation and differentiation, prior to transplantation (2 days *in vitro* prior to transplantation plus period required to recruit host LPCs), experiments investigating mice transplanted with iTEC-MultiRTOC containing donor thymocytes from BM mutated mice could be carried out. Mutated BM could be obtained from mouse models such as: *H2-Aα*$^{-/-}$ (lacking SP4), *β2m*$^{-/-}$ (lacking SP8), *Tcrα*$^{-/-}$ and *Zap70*$^{-/-}$ (lacking all SPs), TCR^- SCID, *Rag1*$^{-/-}$ *and Rag2*$^{-/-}$ (block at DN3) mutant mice which display blocks at various stages of T cell development. As availability of donor

thymocytes is not an issue this was not examined, and other experiments discussed next were prioritised.

5.4. Conclusions

To conclude, it was discovered that iTEC, upon transplantation alongside WT MEFs with or without donor thymocytes, were able to recruit host lymphoid progenitors and support their T cell lineage commitment and development. However, the presence of thymocytes in grafts resulted in improved native thymus-like histology.

From this work, the currently optimum protocol for grafted iTEC-RTOC is multiple RTOC containing iTEC, WT MEFs and donor thymocytes. This is the condition that will be taken forward and discussed in Chapter 6.

Chapter 6: Investigation into the capacity of iTEC-RTOC to reconstitute T cell immunity in athymic *Foxn1$^{G/G}$* mice

6.1. Introduction and aims

The data presented in Chapters 3, 4 and 5 characterised the T cells generated within grafted iTEC-RTOC using flow cytometric analysis but did not analyse the diversity or functionality of the peripheral T cell repertoire that developed in the graft recipients. Bredenkamp and colleagues (Bredenkamp et al., 2014) using the original iTEC protocol, reported that nude (*Foxn1*$^{-/-}$) mice that received iTEC grafts developed peripheral T cells, but showed only limited analyses of three iTEC recipients (CD4, CD8, CD3ε, TCRβ, CD62L, CD44, FOXP3, plus five Vβ-specific antibodies).

A T cell repertoire composed of a pool of T cells that collectively express a diverse array of unique T cell receptors (TCR) is required for a healthy adaptive immune system, in order to allow recognition of the wide variety of non-self-antigens that may be presented to T cells in the context of major histocompatibility complex (MHC) molecules on antigen presenting cells. All TCR chains contain a constant region and a variable region, which is responsible for antigen recognition. The TCRβ chain is encoded by variable (V), diversity (D) and joining (J) genes. VDJ recombination takes place via a combinatorial rearrangement mechanism in which each V gene segment may recombine randomly with each D and J segment to form variable domains, giving a potential combinatorial diversity of 10^{14} (Murugan *et al*., 2012). Random addition or deletion of nucleotides at the junction between the gene segments introduces additional diversity, called junctional diversity (Burtrum *et al*., 1996; Miles, Douek and Price, 2011; Rosati *et al*., 2017), as does recombination with a constant region. Overall, this process of T cell receptor rearrangement results in a highly diverse pool of TCRβ chains, each expressed by an individual T cell clone, that are capable of a wide range of antigen recognition, this is referred to as the primary TCR repertoire. The theoretical diversity of the primary T cell repertoire is 10^{14}.

Each TCR chain contains three complementarity determining regions (CDR1-3) with CDR1 and 2 encoded by V genes and CDR3 by the highly variable region between V and J (Rosati *et al*., 2017). CDR3 is the region of the TCR that comes in direct contact with the peptide antigen of the peptide:MHC complex and its sequence is frequently used to determined T cell clonotypes and therefore the TCR repertoire diversity (Turner *et al*., 2006; Miles, Douek and Price, 2011). T cells most commonly have unique CDR3s unless they have been clonally expanded (Turner *et al*., 2006; Miles, Douek and Price, 2011).

In this chapter, I tested whether iTEC-RTOC generated using the optimised iTEC protocol described in Chapters 3, 4 and 5 could repopulate the periphery of syngeneic athymic mice with a diverse TCR repertoire. The aim of this work was to characterize the iTEC-derived T cell population in greater depth than previously reported, and thus to determine the strengths and weaknesses of the iTEC system for transplantation into T cell deficient hosts.

6.1.A. The *Foxn1$^{G/G}$* nude athymic mouse model

To test whether iTEC-RTOC could repopulate athymic mice with T cells and to characterise the TCR repertoire produced within RTOC based on the optimised iTEC system, a series of transplantation experiments was carried out. For these experiments, knowing that iTEC recruit the host lymphoid progenitors, syngeneic hosts were utilised. Our lab has previously generated a transgenic mouse model (*Foxn1^{G}*) that has a severely hypomorphic *Foxn1* allele (Figure 6.1.A). Heterozygous *Foxn1$^{G/+}$* mice have substantially reduced *Foxn1* expression compared to WT mice. *Foxn1$^{G/G}$* mice are hairless and athymic, effectively phenocopying the *nude* phenotype (Figure 6.1.B). The *Foxn1^{G}* strain was produced and characterized by the Blackburn lab (data not published, Christin Tischner 2010). *Foxn1$^{G/G}$* embryonic thymic lobes were found to consist of K5^{+}K8^{+} TEC, were reduced in size and failed to recruit haematopoietic progenitors to the thymic rudiment. Postnatal *Foxn1$^{G/G}$* mice had alymphoid thymic rudiments, which appeared to consist of TEC arrested in a progenitor state lacking terminal differentiation markers. Therefore, in the experiments described here, *Foxn1$^{G/G}$* nude mice were used as athymic recipients, as they could be generated in house on a C57BL/6J background. Of note is that we were unable to source commercial *Foxn1$^{-/-}$* C56BL/6 mice.

The experiments described below tracked the presence of T cells in the peripheral immune system of these recipient mice over five months, to provide insight into the ability of iTEC-RTOC to repopulate the periphery of the animal with mature T cells, identify the T cell subsets generated and determine the diversity of the TCR repertoires generated, using TCR Vβ region RNA-Seq. These experiments also allowed the simultaneous investigation of the longevity of iTEC-RTOC grafts, which was not tested in the original publication.

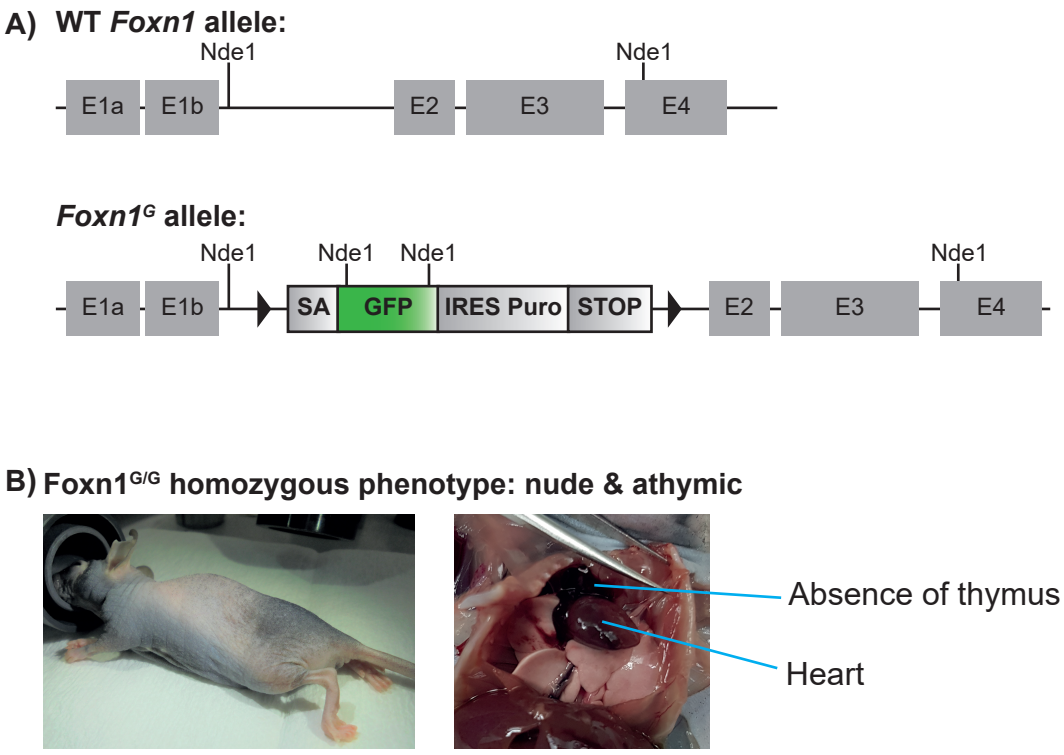


Figure 6.1. *Foxn1*$^{G/G}$ nude athymic mouse model. A) Illustration of *Foxn1*^{G} severe hypomorphic allele. A GFP containing Stop cassette is placed between the promoter and translational start site of the *Foxn1* locus. **B)** Phenotype of *Foxn1*$^{G/G}$ homozygous mice. *Foxn1*$^{G/G}$ homozygous mice are hairless and athymic.