Chapter 4: Investigation of the role of and requirement for Fetal Thymic Mesenchyme (FTM) in iTEC-based RTOC that mimic native thymus organisation and function

4. Introduction and aims

Chapter 3 demonstrated that GFP^+ iTEC harvested at day 18 after iFoxn1 induction in MEFs could be produced in abundance (>20 x 10^6 iTEC / experiment), but that enrichment of this population for the $DLL4^+$ cell fraction was not beneficial for formation and functionality of iTEC-based RTOCs. In addition to iTEC generation, the original iTEC RTOC transplantation system used a number of additional cell-types from murine fetal tissue, namely wild type (WT) MEFs, fetal thymic mesenchyme (FTM), and fetal DN thymocytes for RTOC generation (Bredenkamp *et al*., 2014). However, the original report (Bredenkamp *et al*., 2014) did not establish whether or not these populations were necessary components of the iTEC-RTOC system. The requirement for each population has not been tested and they are limiting in this system due to their scarcity, that limits the translational relevance of the system. Additionally, these cell populations may introduce variation. Thus, the elimination of the need for these populations could reduce variability in an already variable system. Chapter 4 will focus on the requirement of FTM in the system.

Fetal thymic mesenchyme (FTM) is neural-crest derived (rhombomere 6) and has been shown to play an essential role in thymic organogenesis. Previous studies have shown that FTM regulates proliferation and maturation of TEC (Itoi *et al*., 2007; Jenkinson *et al*., 2007; Kioussis *et al*., 2008), and therefore, it is believed to play an important role in generating and maintaining the thymic microenvironments and architecture needed to support thymopoiesis. The mesenchyme present in the fetal thymus is thought to generate the capsule, trabeculae and intrathymic network of fibroblasts and associated extracellular matrix (ECM) that provides some of the organ's structural framework, and produces factors such as integrins, growth factors and cytokines (Suniara, Jenkinson and Owen, 2000). More recently, Nitta and colleagues also demonstrated the importance of medullary fibroblasts for central tolerance induction through provision of an abundance of diverse self-antigens (Nitta

et al., 2020). The interactions between FTM and TEC, and FTM and lymphocytes, remain poorly understood. However, thymic mesenchyme is thought to have a role, together with TEC, in thymocyte development from the DN ($CD4^-CD8^-$) to DP ($CD4^+CD8^+$) stage: thymic lobes lacking FTM displayed poor thymopoiesis from the DN to DP stage even in the presence of mature thymic epithelial cells, suggesting the role of FTM goes beyond just acting on TEC (Anderson, Jenkinson and Moore, 1993; Suniara, Jenkinson and Owen, 2000). Anderson and colleagues reported that FTM could be substituted by a murine fibroblast cell line (3T3), but not soluble factors in 3T3-conditioned medium (Anderson, Jenkinson and Moore, 1993).

The aim of this chapter was to investigate the role of and need for FTM in the iTEC-RTOC system, and subsequently identify key factors produced by FTM that may act on iTEC and test their ability to replace FTM function in RTOC. By establishing whether or not FTM is a necessary input in the iTEC-RTOC system, the data presented provide important information relevant to the development of a transplantable synthetic thymic organ that could be used for clinical purposes.

4.1. Investigation of the role of and requirement for Fetal Thymic Mesenchyme (FTM) in iTEC-RTOC Transplantation

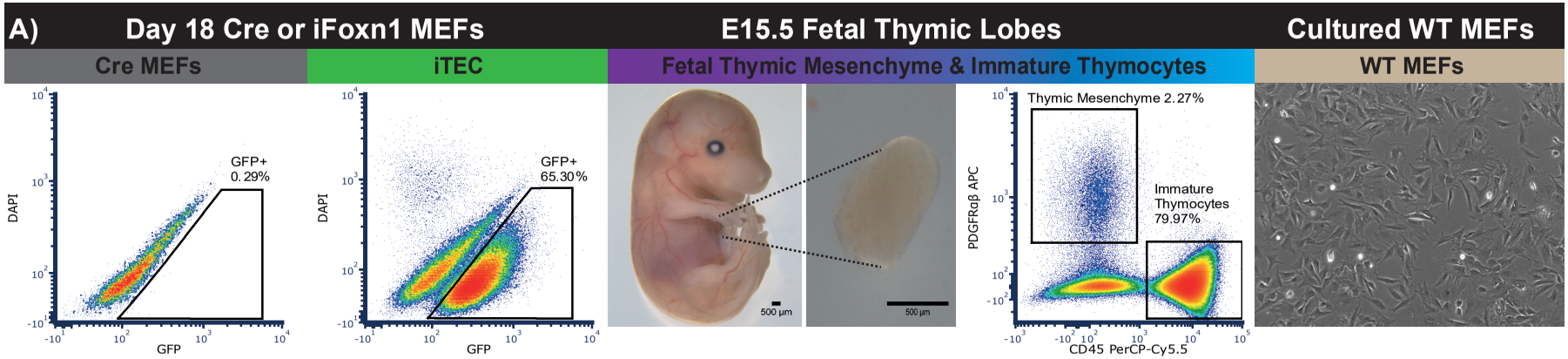
To investigate whether FTM is a necessary component of the iTEC RTOC system, I designed a series of experiments in which iTEC-RTOC, generated with or without FTM, were transplanted into syngeneic hosts. WT MEFs established on the same background as iTEC were used in all conditions, to test whether they could replace FTM. Inclusion of WT MEF in RTOC also improved the reaggregation success rate and ease of transfer from *in vitro* to *in vivo* without disruption of the structures (data not shown). The grafted iTEC-RTOC generated in these experiments were assayed for ability to support thymopoiesis and for architecture, upon recovery at the experimental endpoint.

iTEC were isolated from GFP^+ iFoxn1 MEFs eighteen days after an initial 48-hour period of 4-OHT treatment, using flow cytometry. Cre MEFs at the same timepoint were used as a negative control to control for the effects of tamoxifen on MEFs. Thymic lobes were microdissected from E15.5 C57BL/6J embryos, and digested to single cell suspensions, from which FTM and DNs were purified by flow cytometry. A lineage cocktail was used to exclude cells that were not of interest, including mature T cells, macrophages, dendritic cells, red blood cells, and B cells (Lin = αCD3ε, αCD4, αCD8, NK1.1, GR-1, TER119, αCD11b, αCD11c, B220, all FITC conjugated). FTM were isolated by sorting for $Lin^-PDGFR\alpha\beta^+$ and DNs by sorting for Lin^-CD45^+ cells. WT MEFs were cultured *in vitro* for 48 h prior to the experiment and collected following trypsinisation when needed. Representative FACS plots for obtaining all desired cell types are shown in Figure 4.1.1.A. FTM were limiting for experimental design due to their scarcity, with upward of 50 embryos required to obtain enough FTM for three RTOC.

RTOC for these experiments were established, with the input cell numbers shown in Figure 4.1.1.B. The following conditions were used:

- Test conditions: iTEC only (iTEC + WT MEFs + DN), iTEC + FTM (iTEC + WT MEFs + DN + FTM)
- Negative controls: Cre MEFs (Cre MEFs + WT MEFs + DN), Cre MEFs + FTM (Cre MEFs+ WT MEFs + DN + FTM)
- Positive control: RFTOC (reaggregated fetal thymic organ culture generated from 6 whole dissociated E15.5 thymic lobes + WT MEFs).

RTOC were left to aggregate for two days and were then transplanted under the kidney capsule of syngeneic mice and left for two weeks.



B) RTOC Conditions				
iTEC	**iTEC + FTM**	**Cre MEFs**	**Cre MEFs + FTM**	**RFTOC**
200,000 iTEC	200,000 iTEC	200,000 Cre MEFs	200,000 Cre MEFs	6 dissociated E15.5 thymic lobes
100,000 WT MEFs	100,000 WT MEFs	100,000 WT MEFs	100,000 WT MEFs	200,000 WT MEFs
100,000 DN Thymocytes	100,000 DN Thymocytes	100,000 DN Thymocytes	100,000 DN Thymocytes	
	50,000 FTM		50,000 FTM	

Figure 4.1.1. Experimental strategy for investigating whether FTM is required in iTEC-RTOC. A) Cre MEFs were obtained from Day 18 Cre MEFs in culture. iTEC are isolated from GFP+ Day 18 iFoxn1 MEFs. Fetal thymic mesenchyme and DN thymocytes were collected from dissociated E15.5 WT thymic lobes. WT MEFs are cultured and harvested. **B)** Detailed description of cells and numbers used in each condition.

4.1.A. Influence of FTM on thymocyte development in iTEC-RTOC

At the experimental endpoint of two weeks, grafts were recovered from the recipient mice. Thymocytes were released from the grafts and processed for FACS analysis described in Materials and Methods. Initially, thymopoiesis was investigated in terms of progression from DN ($CD4^-CD8^-$) to DP($CD4^+CD8^+$) and DP to SP $CD4^+$ (SP4) and SP $CD8^+$ (SP8); and the generation of $CD3\varepsilon^+TCR\beta^+$ cells. A lineage cocktail was not used in this experiment so the $CD45^+$ gate may contain lineages other than T cells.

Representative FACS plots and gating hierarchy are shown in Figures 4.1.2 - 4.1.6 for iTEC, iTEC+FTM, Cre MEFs, Cre MEFs+FTM, RFTOC conditions, respectively. CD4 and CD8 plots for all the RTOC examined in this section are shown in Figure 4.1.7 to display variability. Cell percentages rather than absolute numbers are shown, as the BD LSRFortessa™ does not perform an absolute cell count when analyzing and the grafts were too small to perform an accurate manual cell count. The collated data from three independent experiments are shown in Figure 4.1.8. Of note is that in smaller grafts, such as the negative controls, the percentage of populations such as DN, SP4 and SP8 can appear high even though cell number is low (as evidenced by cell density in the flow cytometric analysis plots).

The data presented show that progression of DN to DP and SP subsets was supported in all of the test condition grafts (iTEC only; iTEC+FTM) and in the positive control grafts, but not in any of the negative control conditions. For negative controls, this conclusion is based on the absence of DP T cells, which occur only during T cell development in the thymus (Koch and Radtke, 2011).

These analyses also showed that iTEC+FTM-RTOC performed better than iTEC-only RTOC, as evidenced by the increased percentage of DP T cells in the iTEC+FTM grafts. iTEC+FTM-RTOC were found to have statistically significantly higher levels of DP thymocytes than iTEC-RTOC as shown by an unpaired t-test ($p=0.0004$). Additionally, visual observation together with the cell densities evident in the FACS plots show that the iTEC+FTM grafts typically contained more cells than the iTEC-only grafts. However, iTEC-RTOC were still able to support the generation of all T cell subsets in the absence of FTM. The data for each biological replicate are shown in Table 4.1.1. Note that replicate B in the Cre MEFs control contained 22% DP cells; The latter could be explained by the possibility that some DNs may have contained some thymocytes that had already undergone β-selection and then

progressed to DP. However, when investigating cell density plots (data not shown) few cells are present (~12) in the DP quadrant and this high proportion is also attributed to the overall small number of cells in this replicate.

To conclude, thymocyte differentiation occurs in the absence of FTM, but FTM influences thymocyte development in terms of numbers and proportions of different T cell subsets. However, whether this effect reflects increased progression from DN to DP stage or proliferation of thymocytes at one or more stages of development remains unknown. These data also show that WT MEFs do not compensate for the absence of FTM. RTOC in the absence of WT MEFs was not investigated.

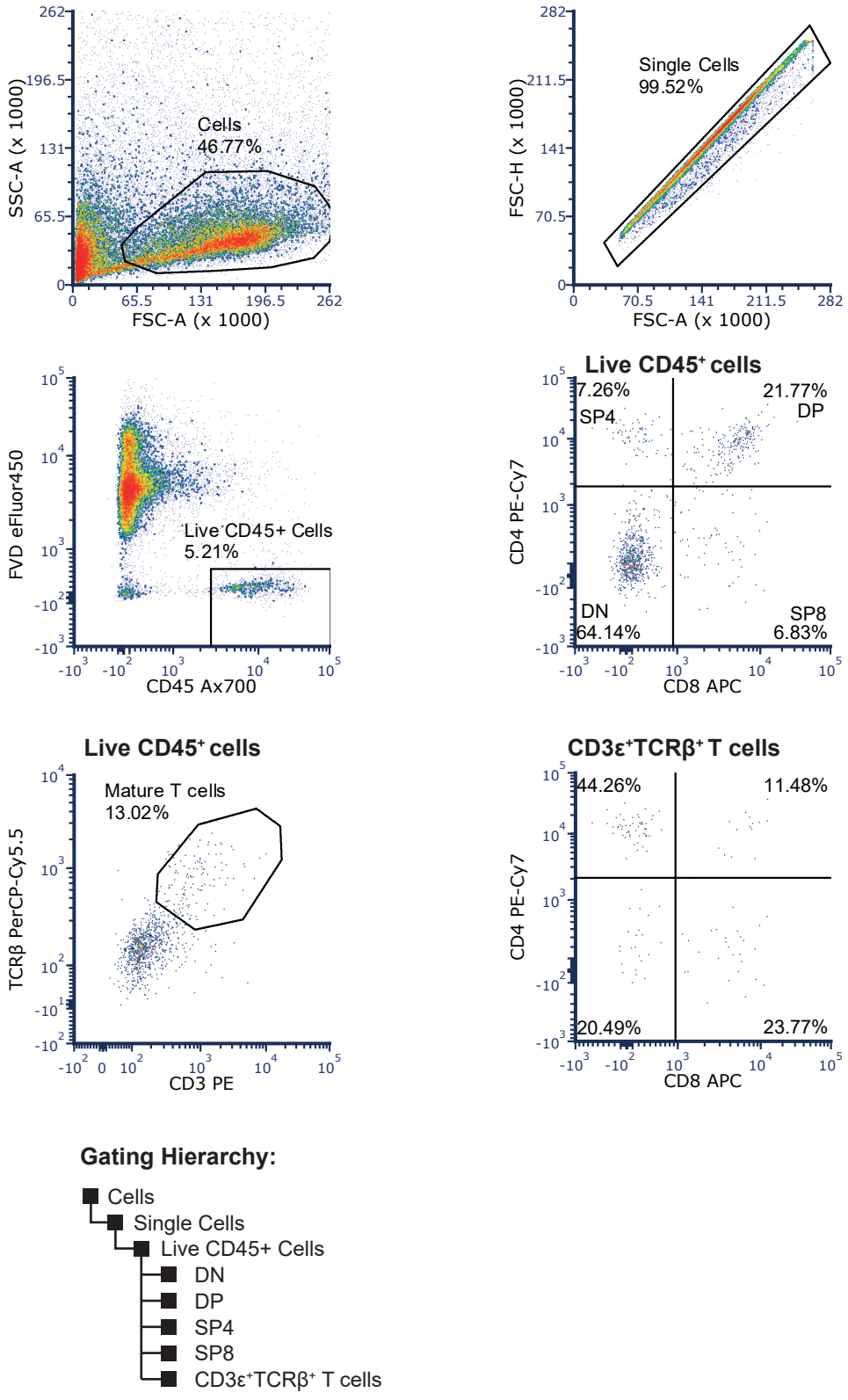


Figure 4.1.2. Thymopoiesis in iTEC-RTOC grafts recovered 2 weeks post grafting. Representative FACS plots for iTEC-RTOC 2 weeks post transplantation. Transplant recipients received one RTOC consisting of 200,000 iTEC + 100,000 DNs + 100,000 WT MEFs.

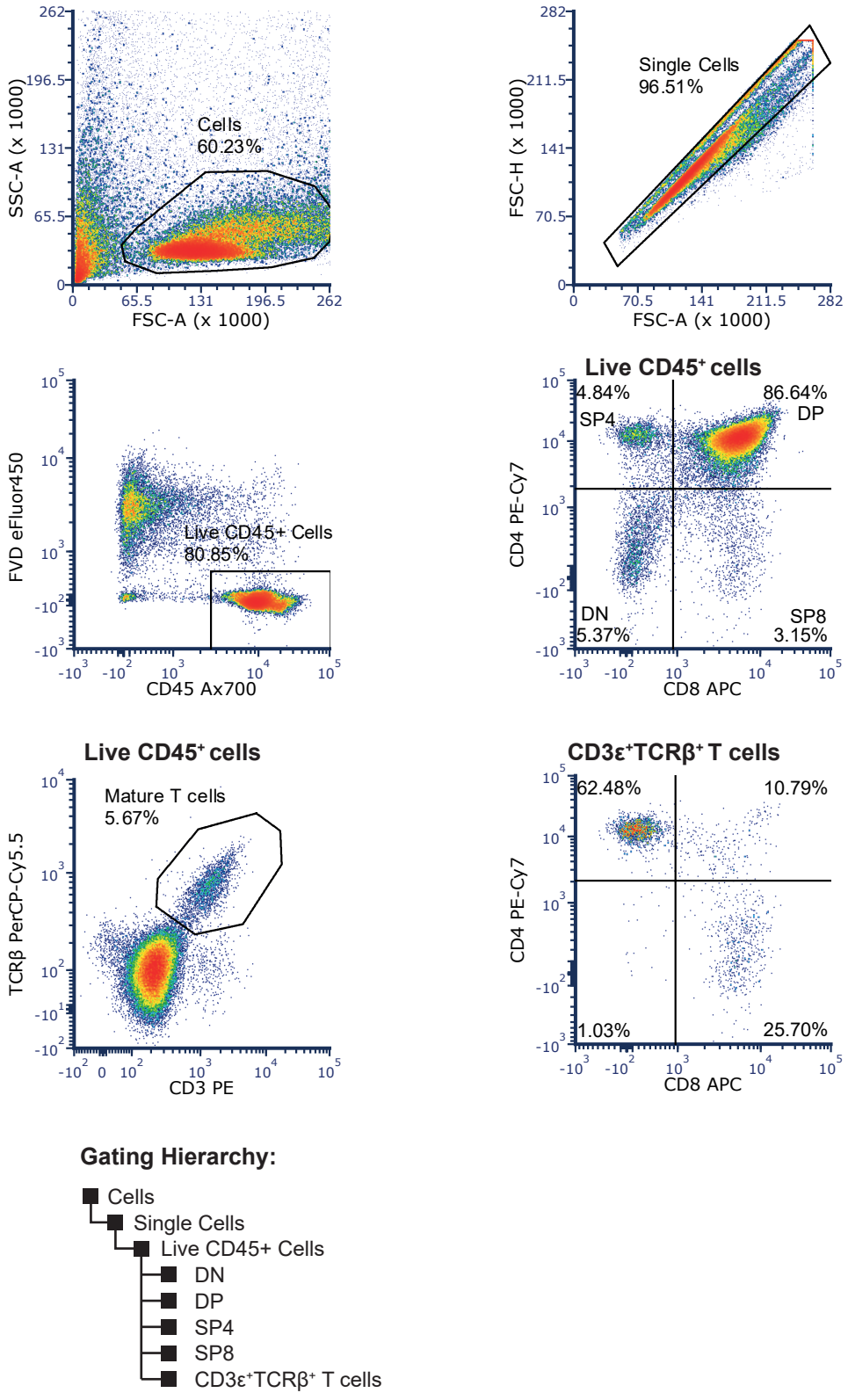


Figure 4.1.3. Thymopoiesis in iTEC+FTM-RTOC grafts recovered 2 weeks post grafting. Representative FACS plots for iTEC+FTM-RTOC 2 weeks post transplantation. Transplant recipients received one RTOC consisting of 200,000 iTEC + 100,000 DNs + 100,000 WT MEFs + 50,000 Fetal Thymic Mesenchyme (FTM).

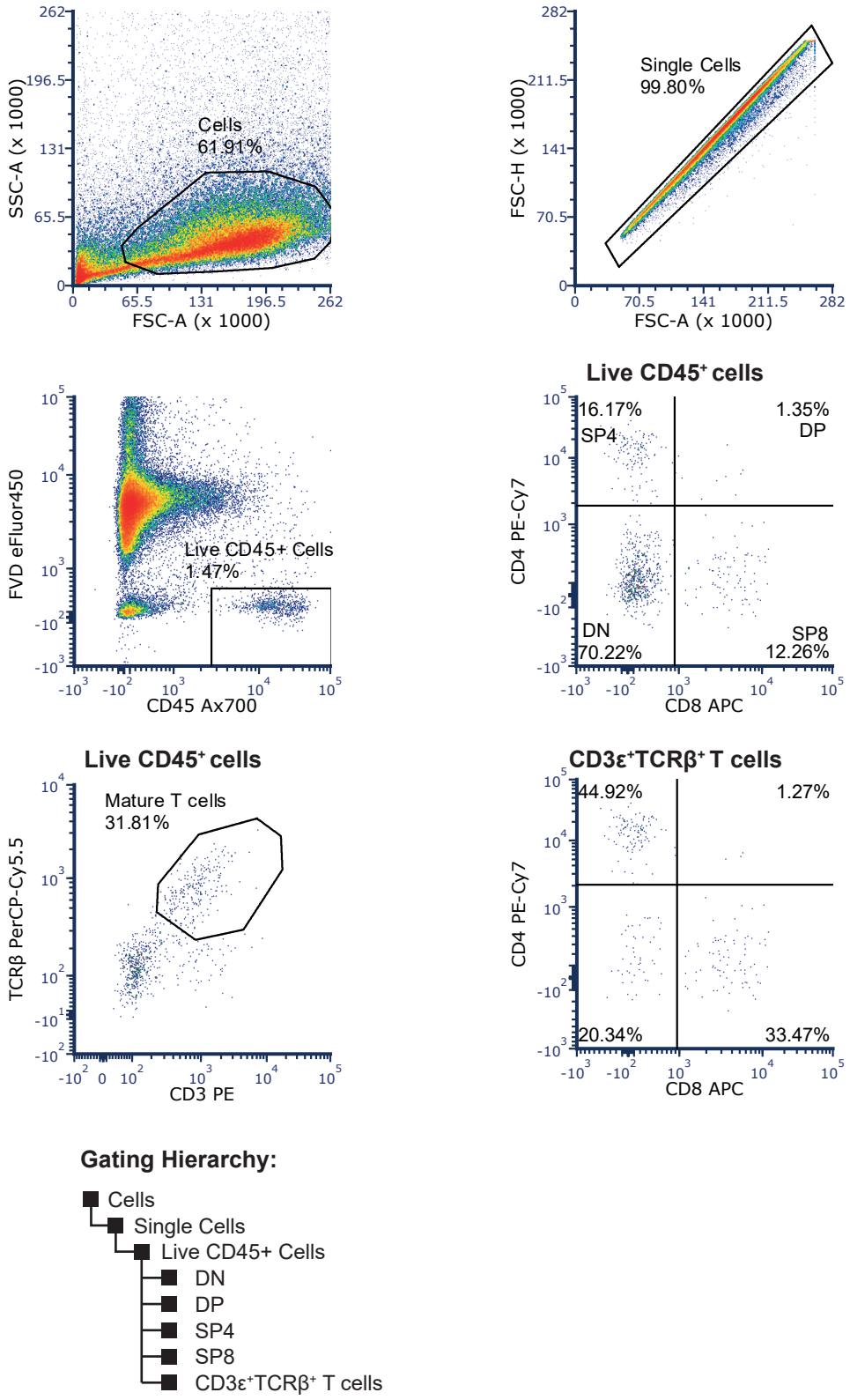


Figure 4.1.4. Thymopoiesis in Cre MEF-RTOC grafts recovered 2 weeks post grafting. Representative FACS plots for Cre MEF-RTOC 2 weeks post transplantation. Transplant recipients received one RTOC consisting of 200,000 Cre MEFs + 100,000 DNs + 100,000 WT MEFs.

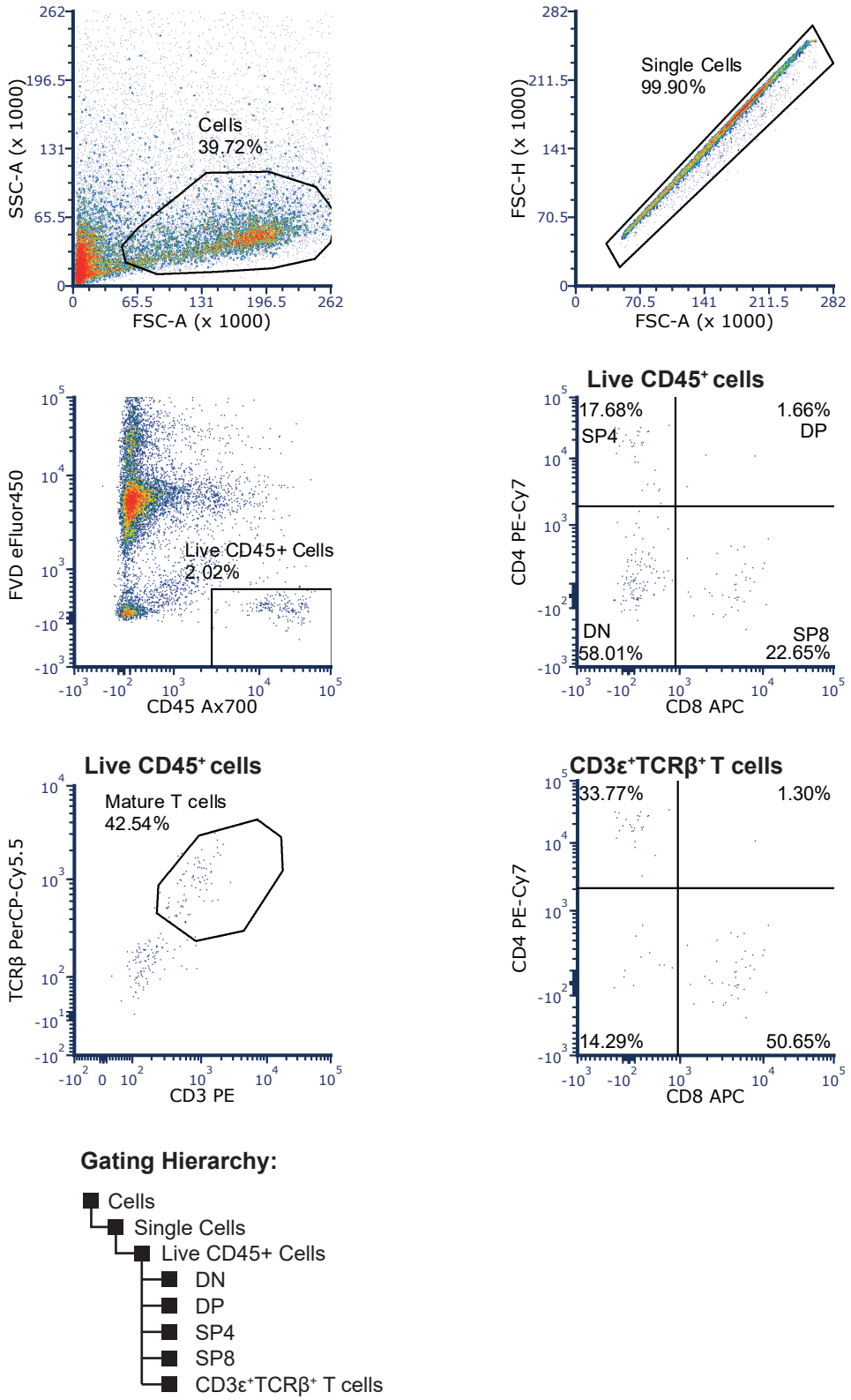


Figure 4.1.5. Thymopoiesis in Cre MEF+FTM-RTOC grafts recovered 2 weeks post grafting. Representative FACS plots for Cre MEF+FTM-RTOC 2 weeks post transplantation. Transplant recipients received one RTOC consisting of 200,000 Cre MEFs + 100,000 DNs + 100,000 WT MEFs + 50,000 Fetal Thymic Mesenchyme (FTM).

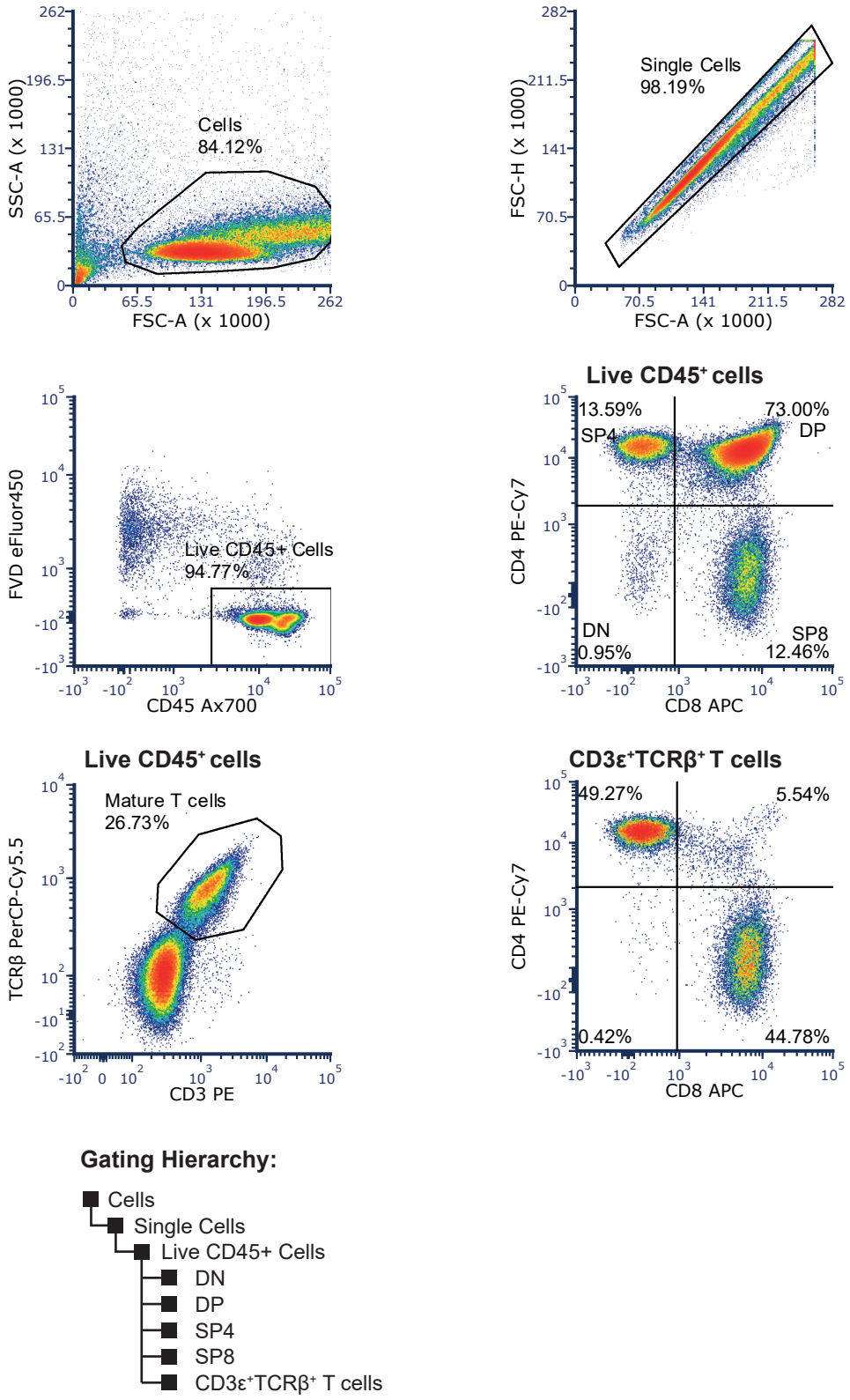


Figure 4.1.6. Thymopoiesis in RFTOC grafts recovered 2 weeks post grafting.
Representative FACS plots for positive control RFTOC 2 weeks post transplantation. Transplant recipients received one RTOC consisting of 6 dissociated E15.5 thymic lobes + 200,000 WT MEFs.

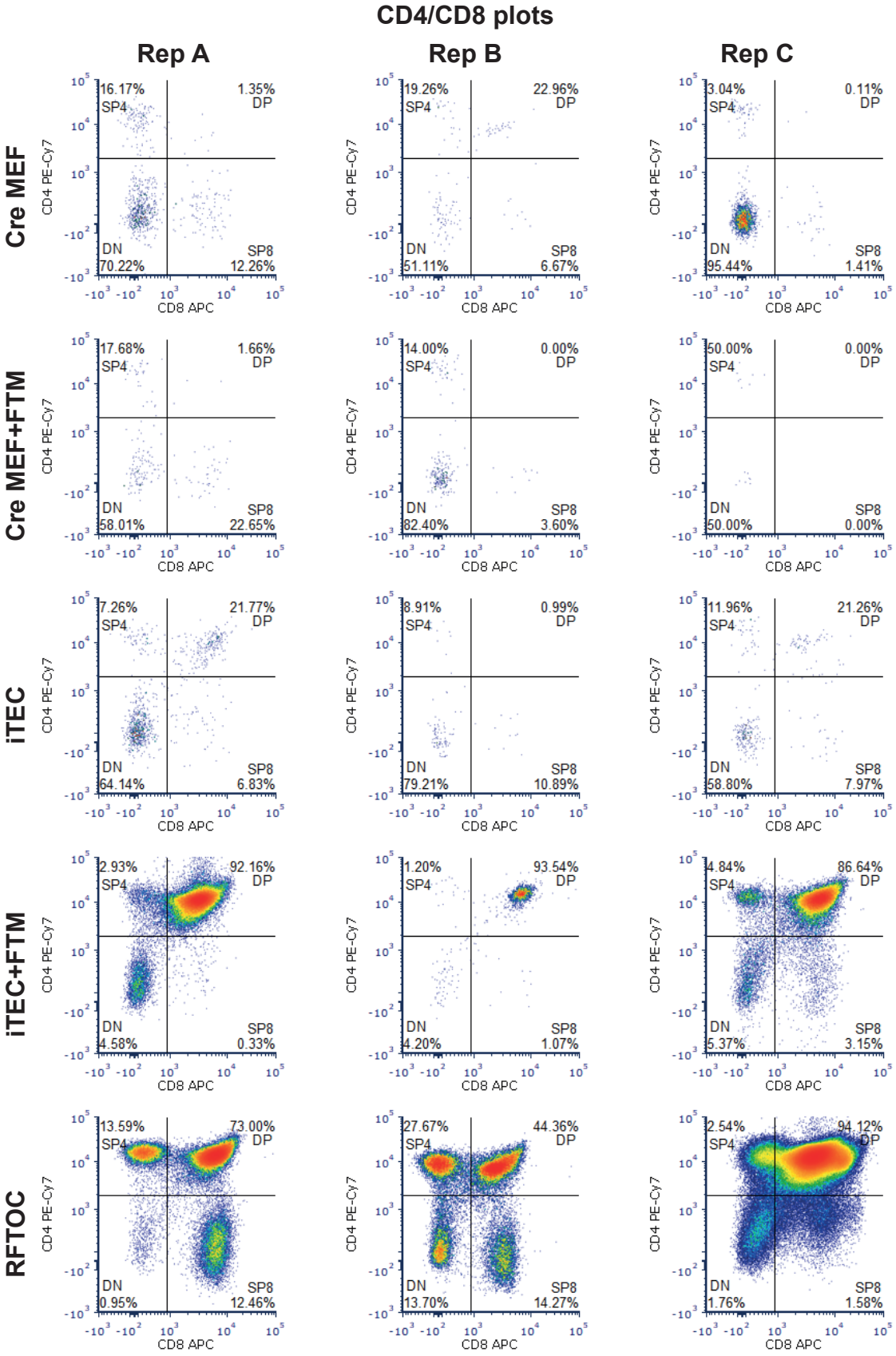
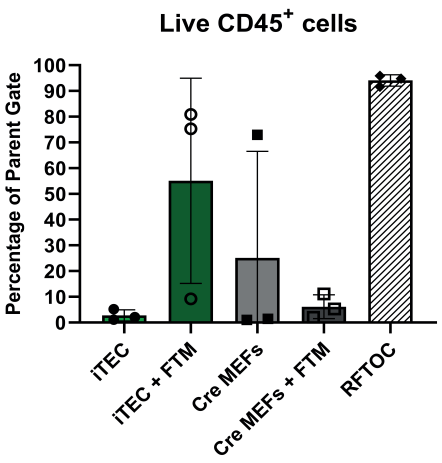
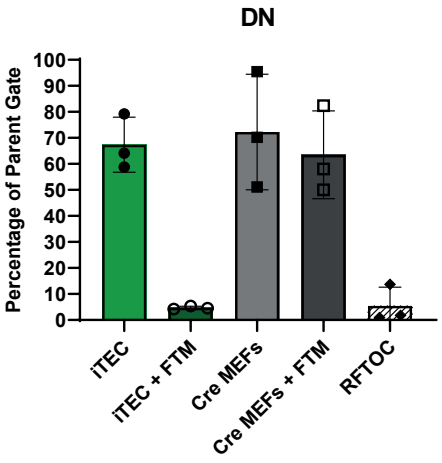


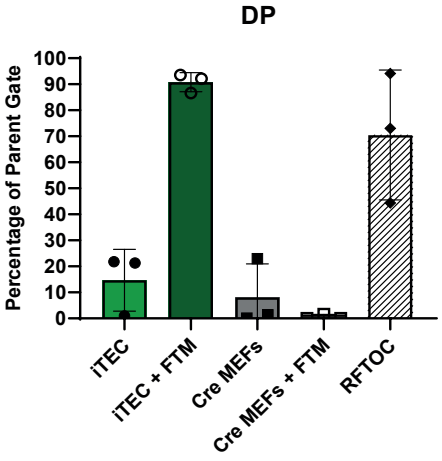
Figure 4.1.7. RTOC CD4/CD8 FACS plots for each experiment. Collated FACS plots of CD4 and CD8 T cell profiles of each RTOC condition for each of the three independent experiments.

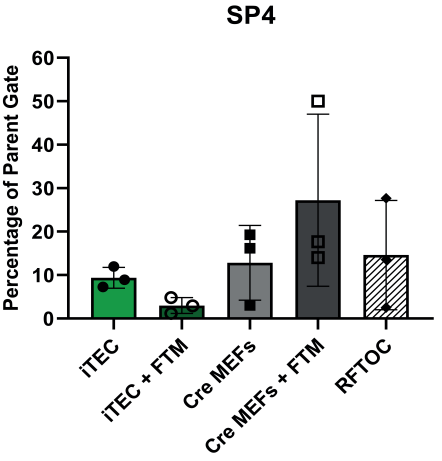
Condition		CD45$^+$ cells	DN	DP	SP4	SP8	CD3ε$^+$TCRβ$^+$
iTEC	Rep A	5.21	64.14	21.77	7.26	6.83	13.02
	Rep B	2.12	58.8	21.26	11.96	7.97	18.6
	Rep C	1.09	79.21	0.99	8.91	10.89	19.8
	Mean	2.81 ±2.14	67.38 ±10.58	14.67 ± 11.85	9.38 ±2.38	8.56 ±2.09	17.14 ±3.62
iTEC + FTM	Rep A	75.31	4.58	92.16	2.93	0.33	3.39
	Rep B	80.85	5.37	86.64	4.84	3.15	5.67
	Rep C	9.18	4.2	93.54	1.2	1.07	2.66
	Mean	55.11 ±39.88	4.72 ±0.60	90.78 ±3.65	2.99 ±1.82	1.52 ±1.46	3.91 ±1.57
Cre	Rep A	1.47	70.22	1.35	16.17	12.26	31.81
	Rep B	1.01	51.11	22.96	19.26	6.67	26.67
	Rep C	72.96	95.44	0.11	3.04	1.41	4.02
	Mean	25.15 ±41.41	72.26 ±22.24	8.14 ±12.85	12.82 ±8.61	6.78 ±5.43	20.83 ±14.79
Cre + FTM	Rep A	2.02	58.01	1.66	17.68	22.65	42.54
	Rep B	11.14	82.4	0	14	3.6	16
	Rep C	5.28	50	0	50	0	43.75
	Mean	6.15 ±4.62	63.47 ±16.88	0.55 ±0.96	27.23 ±19.81	8.75 ±12.17	34.10 ±15.68
RFTOC	Rep A	94.77	0.95	73	13.59	12.46	26.73
	Rep B	95.91	13.7	44.36	27.67	14.27	36.14
	Rep C	91.62	1.76	94.12	2.54	1.58	4.42
	Mean	94.10 ±2.22	5.47 ±7.13	70.49 ±24.97	14.60 ±12.59	9.44 ±6.86	22.43 ±16.29

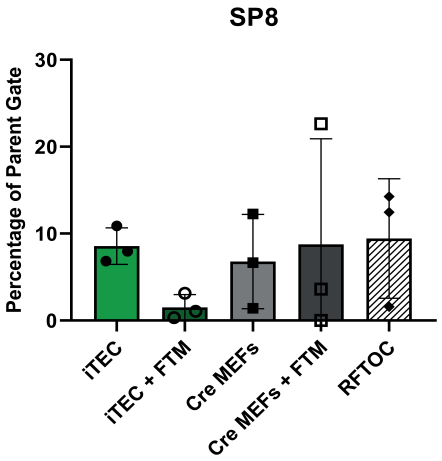
Table 4.1.1. Cell percentages of parent gate for T cell populations recovered in grafts in the presence or absence of FTM. Detailed account of cell percentages of populations for three biological replicates per condition, with mean ± SD. Rep, replicate.

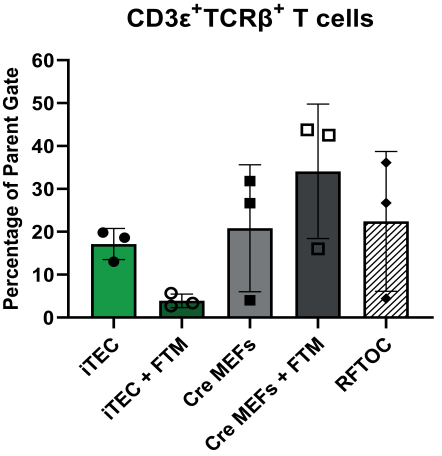

Live CD45+ cells
Percentage of Parent Gate
100
90
80
70
60
50
40
30
20
10
0
iTEC
iTEC + FTM
Cre MEFs
Cre MEFs + FTM
RFTOC


DN
Percentage of Parent Gate
100
90
80
70
60
50
40
30
20
10
0
iTEC
iTEC + FTM
Cre MEFs
Cre MEFs + FTM
RFTOC


DP
Percentage of Parent Gate
100
90
80
70
60
50
40
30
20
10
0
iTEC
iTEC + FTM
Cre MEFs
Cre MEFs + FTM
RFTOC


SP4
Percentage of Parent Gate
60
50
40
30
20
10
0
iTEC
iTEC + FTM
Cre MEFs
Cre MEFs + FTM
RFTOC


SP8
Percentage of Parent Gate
30
20
10
0
iTEC
iTEC + FTM
Cre MEFs
Cre MEFs + FTM
RFTOC


CD3ε+TCRβ+ T cells
Percentage of Parent Gate
60
50
40
30
20
10
0
iTEC
iTEC + FTM
Cre MEFs
Cre MEFs + FTM
RFTOC

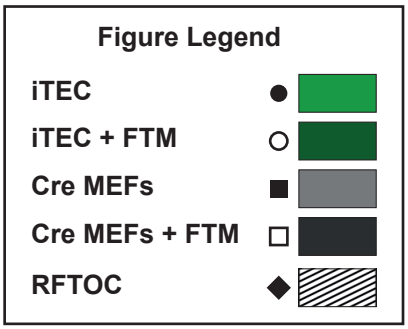


Figure 4.1.8. Cell percentages of thymocytes present in grafts 2 weeks *in vivo* in the presence or absence of FTM. Each datapoint represents one biological replicate. T cells ($CD3\varepsilon^{+}TCR\beta^{+}$), DN ($CD4^{-}CD8^{-}$), DP ($CD4^{+}CD8^{+}$), SP4($CD4^{+}$), SP8 ($CD8^{+}$). DP percentages are indicative of success rate of undergoing thymopoiesis in the recovered grafts. Graphs display mean±SD. iTEC (200,000 iTEC + 100,000 WT MEFs + 100,000 DNs), iTEC + FTM (200,000 iTEC + 100,000 WT MEFs + 100,000 DNs + 50,000 FTM), Cre MEFs (200,000 Cre MEFs + 100,000 WT MEFs + 100,000 DNs), Cre MEFs+ FTM (200,000 Cre MEFs + 100,000 WT MEFs + 100,000 DNs + 50,000 FTM), RFTOC (6 dissociated lobes + 200,000 WT MEFs). N=3 independent biological experiments.

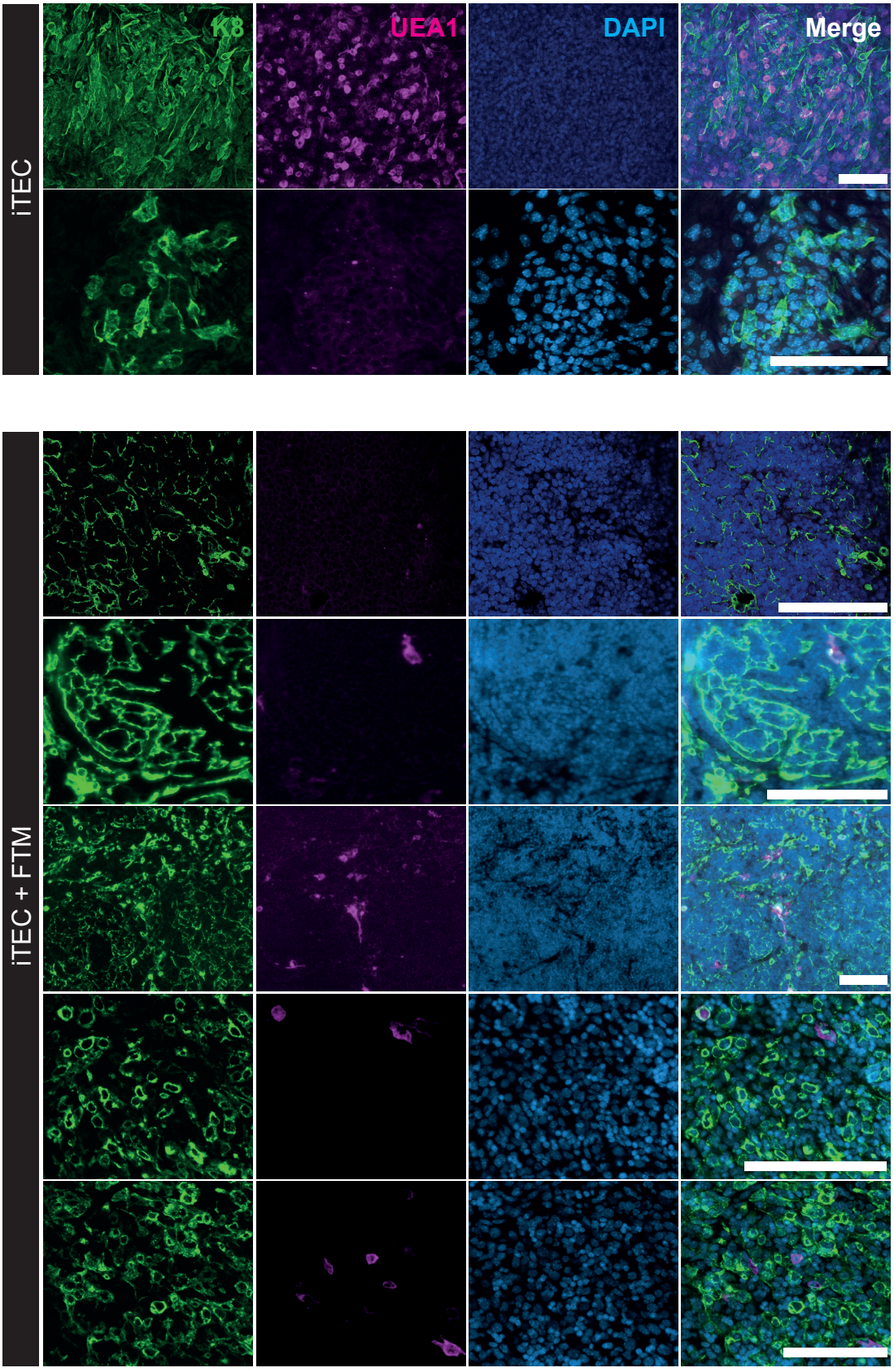
4.1.B. Influence of FTM on formation of cortical and medullary regions in grafted iTEC-RTOC

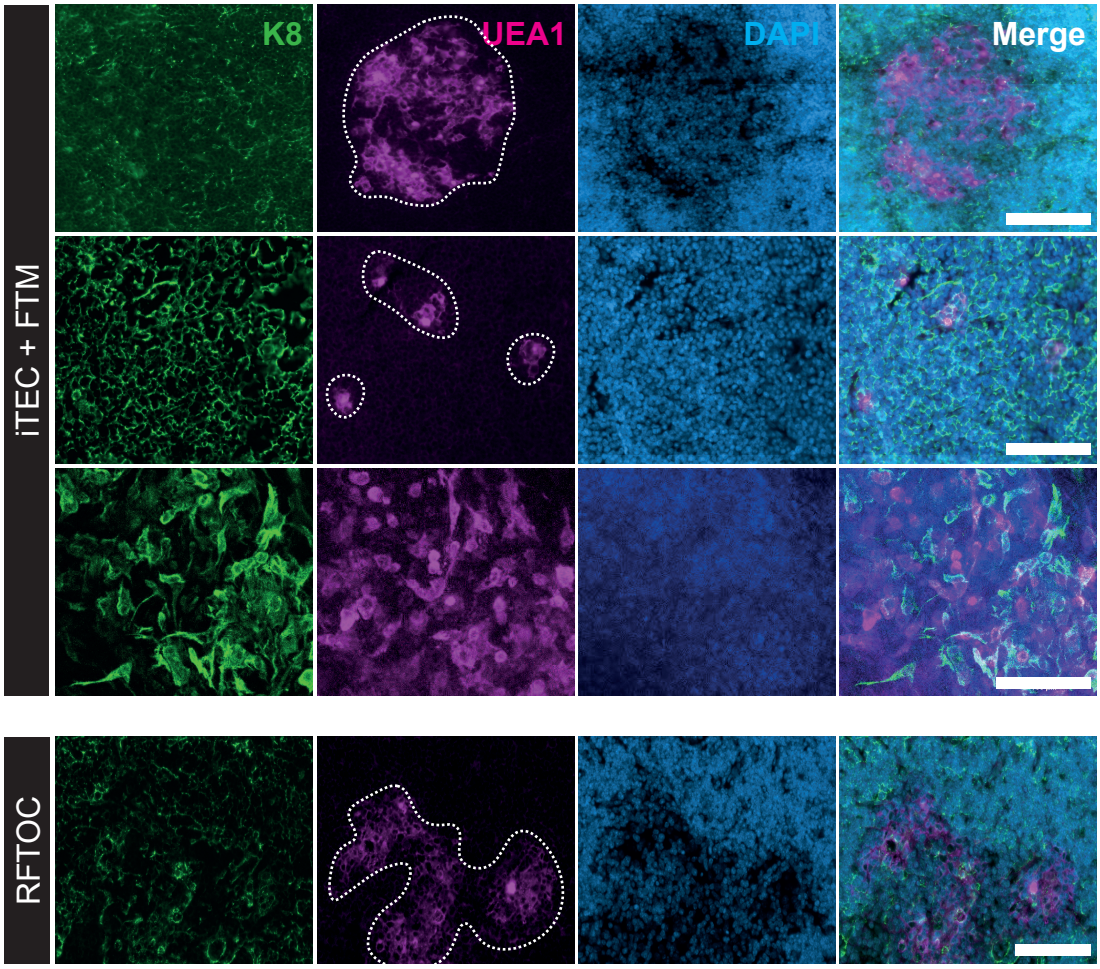
The architecture of the grafts recovered after two weeks was also examined. For the iTEC condition two biological replicates, and for the iTEC+FTM condition four biological replicates, with eight technical replicates in total, were analysed.

As FTM is known to play a role in TEC development (Revest *et al*., 2001), the presence of cortical and medullary iTEC and of clear cortical and medullary regionalisation after two weeks *in vivo* was determined (Figure 4.1.9). For the latter, frozen sections were stained with anti-cytokeratin 8 (K8: green; an epithelial cell marker associated with cTEC (Klug *et al*., 1998, 2002; Danzl *et al*., 2014) and Ulex Europaeus Agglutinin-1 (UEA1: magenta), a lectin that binds mTEC (Klug *et al*., 1998; Kuraguchi *et al*., 2006; Lomada *et al*., 2007). DAPI was also included to show the overall cellularity of the grafts. Cre MEFs or Cre MEFs+FTM controls were not analysed as the recovered grafts were too small to recover and/process for sectioning.

In the iTEC only condition, both recovered grafts contained $K8^+$ cells, but only one contained $UEA1^+$ cells. In the iTEC+FTM condition seven of the eight grafts analysed contained both $K8^+$ and $UEA1^+$ cells and two out of eight showed organisation into cortical and medullary regions (see Figure 4.1.9; medullary regions outlined in white dotted line).

In the RTOC that exhibited cortex:medulla organisation, the distribution of additional TEC markers was also analysed (Figure 4.1.10). Cytokeratin 14 (K14) is preferentially expressed by medullary TEC (Klug *et al*., 1998; Kuraguchi *et al*., 2006; Lomada *et al*., 2007): in the native thymus, medullary islands express high levels of K14 that co-stain with $UEA1^+$. This pattern of K14 expression was observed in one of two iTEC+FTM RTOC analysed (Figure 4.1.10.a, second row). The thymoproteasome β5t (green) was also analysed, as a marker of cTEC functionality. Cortical regions in the thymus express low levels of major histocompatibility complex class II (MHCII) molecules and medullary regions express high levels. iTEC in the recovered grafts were found to stain for β5t and MHCII, however, MHCII high ($MHCII^{hi}$) regions characteristic of the native thymic medulla were not observed in the recovered iTEC+FTM graft examined (Figure 4.1.10.b).


iTEC
K8
UEA1
DAPI
Merge
iTEC + FTM



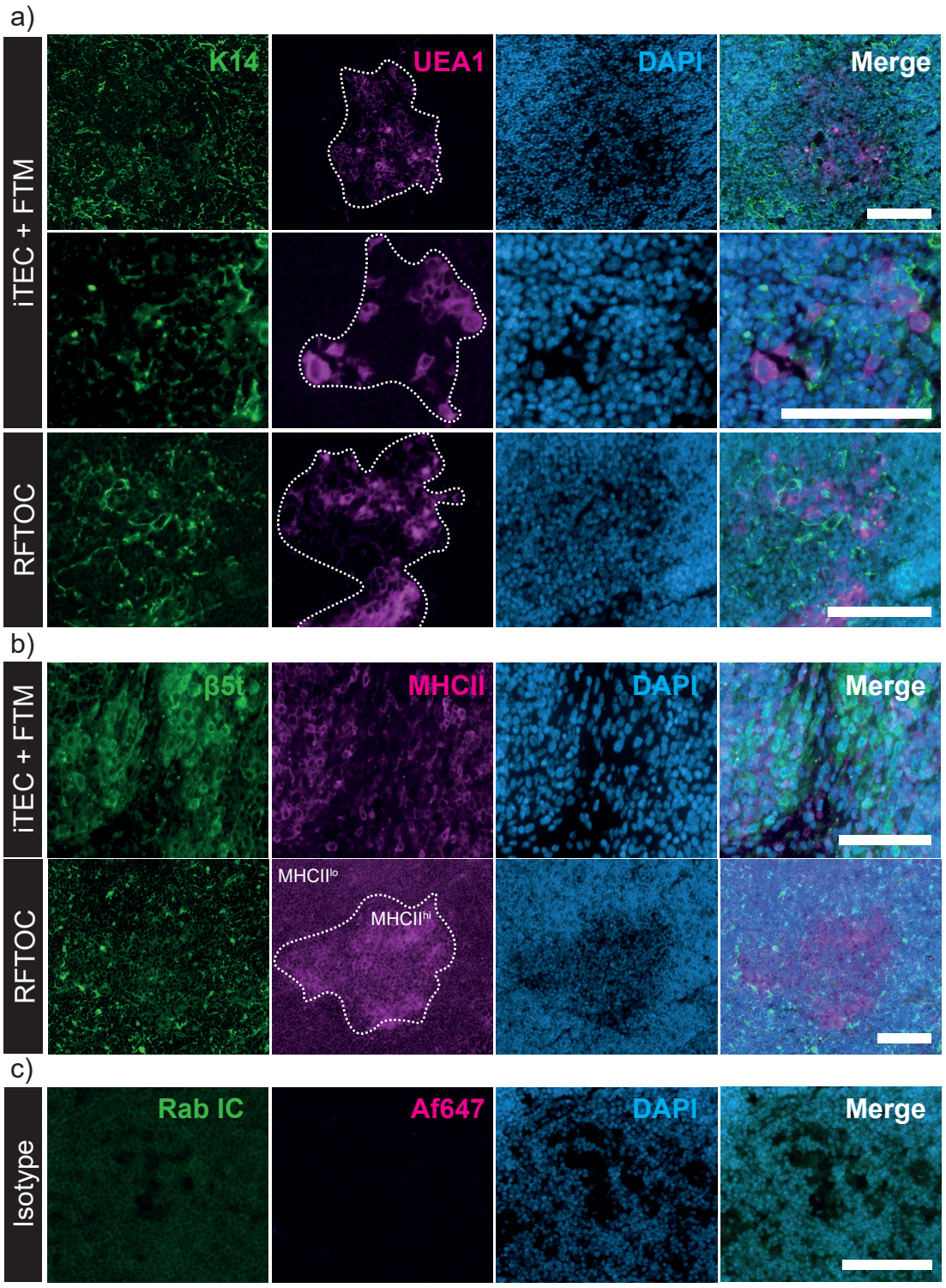
Grafts where UEA1+ cells present:

iTEC	1/2 grafts
iTEC + FTM	7/8 grafts

Grafts where medullary region organisation present:

iTEC	0/2 grafts
iTEC + FTM	2/8 grafts

Figure 4.1.9. Immunohistochemistry staining of recovered grafts two weeks *in vivo* in the presence or absence of FTM. Grafts were stained with cytokeratin 8-K8 (green) for cortical regions, UEA1 (magenta) for medullary regions and DAPI (blue) for cell nucelus. For each condition, representative Regions of interest (ROI) were selected to show where distinct cortical and medullary regions had formed in the graft. Positive controls include recovered RFTOC. iTEC indicates where the host received an iTEC-RTOC and iTEC + FTM indicates where the host received an iTEC+FTM-RTOC. For the iTEC condition N=2 independent experiments. For the iTEC + FTM condition N=4 independent experiments with a total of 8 grafts examined. Each row represents a separate graft. 100 μm scale bar.



UEA1^{+} cells co-localising with K14^{hi} regions:
iTEC + FTM 1/2 grafts

MHCII^{hi} regions occuring:
iTEC + FTM 0/1 grafts

Figure 4.1.10. Immunohistochemistry of additional markers in successfully organised recovered grafts 2 weeks *in vivo* in the presence of FTM. a) Grafts were stained with cytokeratin 14-K14 (green) and UEA1 (magenta) for medullary regions and DAPI (blue) for cell nucleus. **b)** Grafts were stained with β5t (green) for cortical regions and MHCII (magenta) for cortical regions ($MHCII^{lo}$), medullary regions ($MHCII^{hi}$) and DAPI (blue) for cell nucleus. **c)** Negative isotype control. Medullary regions are outlined in a white dotted line. Positive controls include RFTOC. N=1 biological experiment with 2 grafts examined. 100 μm scale bar.

4.1.C. The absence of FTM can be partially compensated for by increasing input iTEC cell numbers

As FTM is known to induce TEC proliferation and maturation (Jenkinson, Jenkinson and Anderson, 2003), I hypothesised that the effects of FTM observed above might be due at least partially to its effects on iTEC. Therefore, I tested whether the absence of FTM could be compensated by increasing iTEC numbers. Due to the technical failure rate associated with RTOC production, a backup system whereby multiple RTOC (MultiRTOC) were transplanted into each recipient was also tried. This MultiRTOC approach would also ensure the prevention of necrotic cores associated with large organoids prior to vascularisation.

Thus, each recipient was transplanted with three iTEC-RTOC. This system will be referred to from here on out as iTEC-MultiRTOC (3 RTOC each consisting of 200,000 iTEC + 100,000 WT MEFs + 100,000 DN) and was compared to iTEC and iTEC+FTM-RTOC. Of note is that iTEC-MultiRTOC grafts typically appeared to fuse together and were typically recovered as a single large graft. In cases where this had not occurred, they were pooled together for analysis.

Representative FACS plots for the iTEC-MultiRTOC condition are shown in Figure 4.1.11. Three independent experiments with two animals transplanted per experiment were performed for the thymopoiesis analysis (Table 4.1.2). CD4 and CD8 plots for each experiment are shown in Figure 4.1.12. The iTEC-MultiRTOC system was compared to iTEC and iTEC+FTM-RTOC generated as described previously (Figure 4.1.13). The iTEC-MultiRTOC system showed an increased proportion of DP thymocytes (mean, 46.62% DP T cells) compared to the previous iTEC-RTOC condition (mean, 14.67% DP T cells) but this was lower than in the iTEC+FTM condition (mean, 90.78% DP T cells). These results indicate that the absence of FTM can be at least partially compensated by increasing iTEC numbers upon transplantation. It is important to note however that statistical significance between iTEC-MultiRTOC and iTEC-RTOC in supporting DP T cell development, with regards to cell percentages, was not achieved ($p=0.1167$).

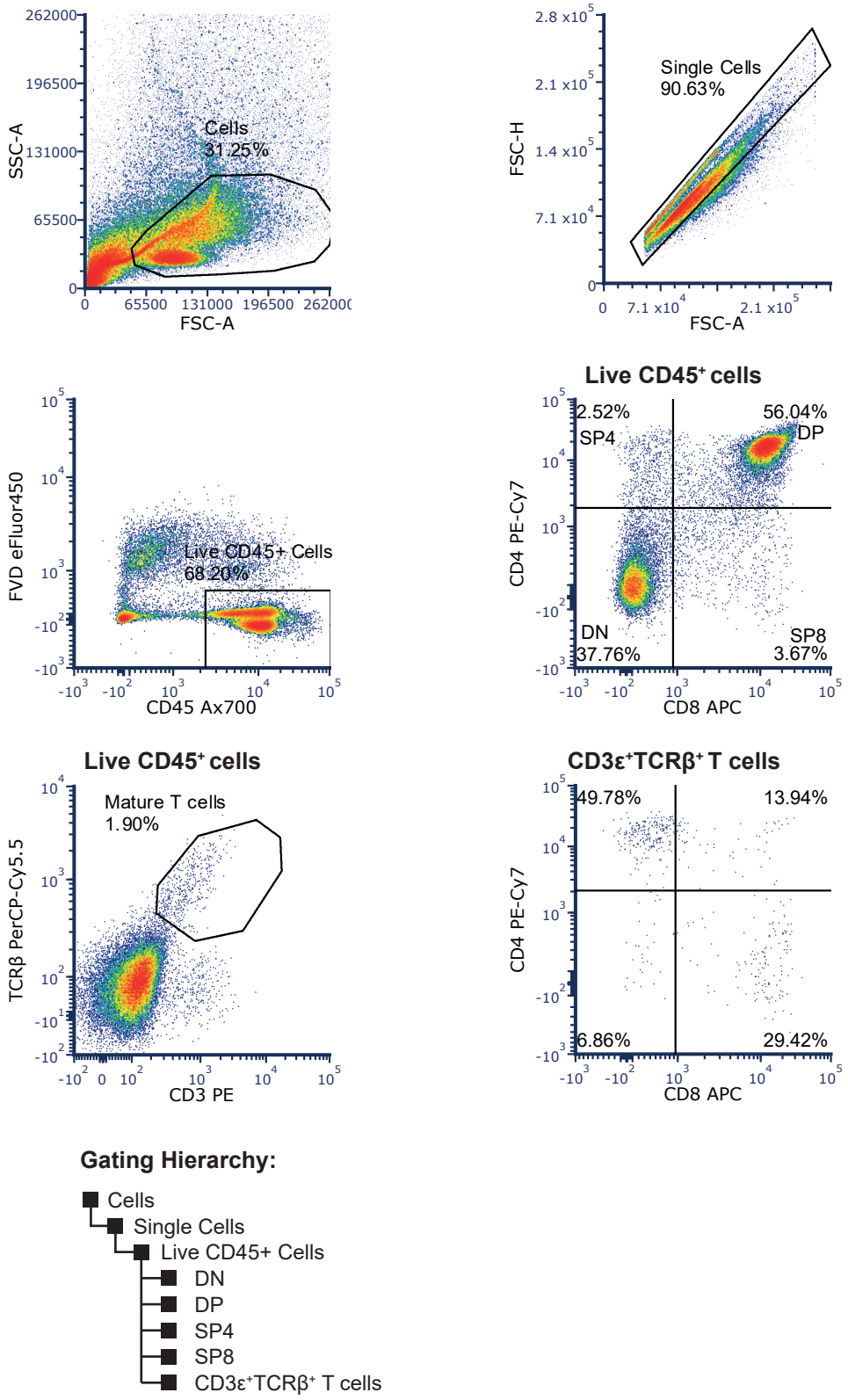


Figure 4.1.11. Thymopoiesis in iTEC-MultiRTOC grafts recovered 2 weeks post grafting. Representative FACS plots for iTEC-MultiRTOC 2 weeks post transplantation. Transplant recipients received three RTOC each consisting of 200,000 iTEC + 100,000 DNs + 100,000 WT MEFs.

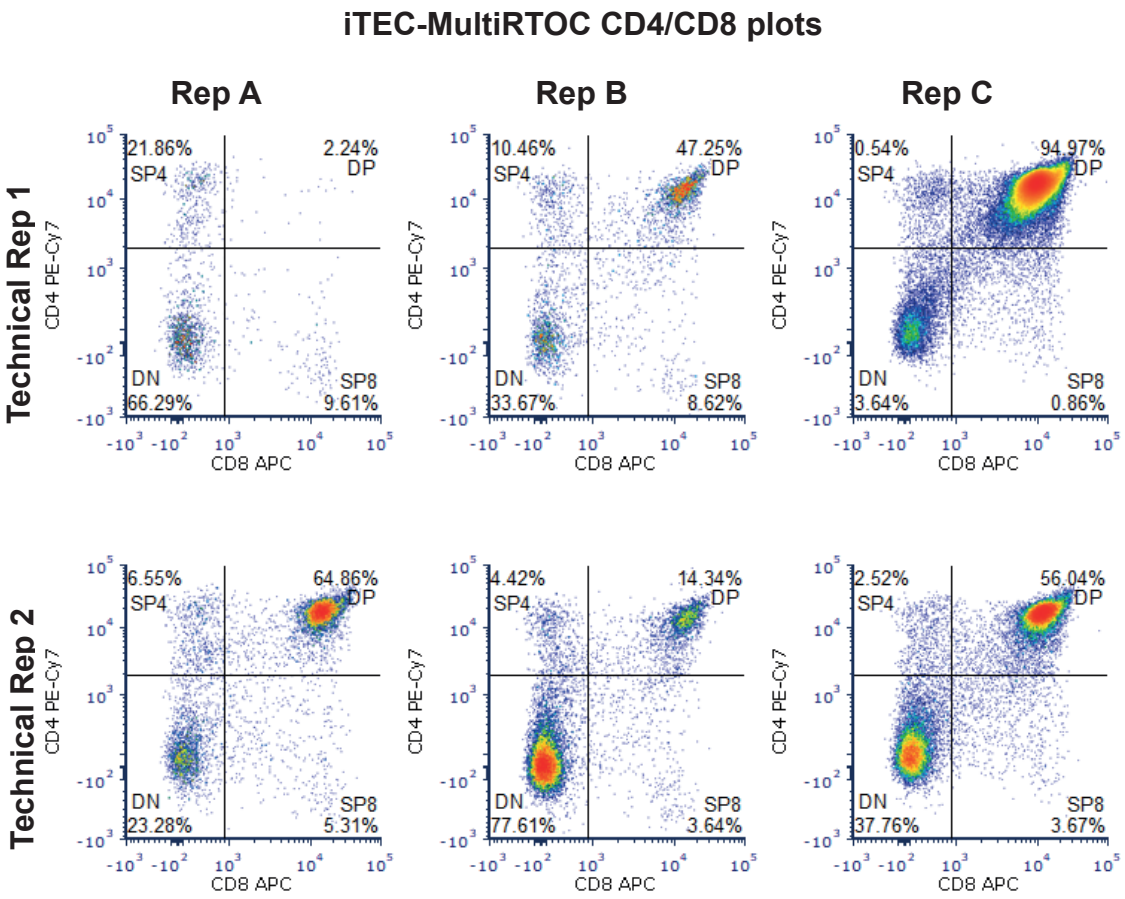
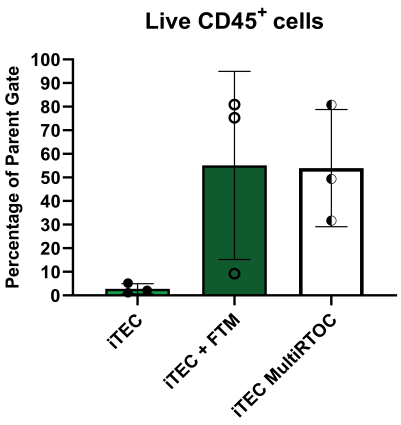
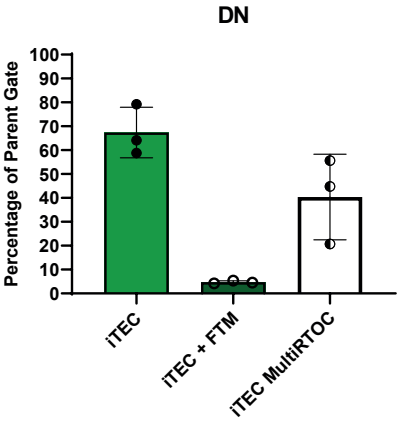


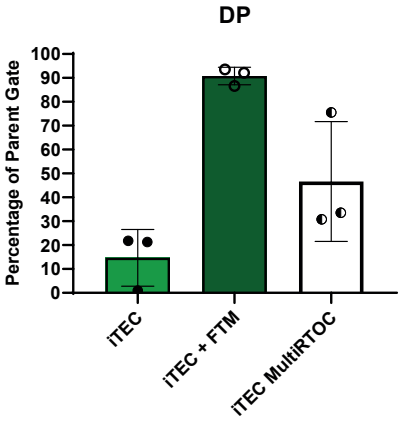
Figure 4.1.12. iTEC-MultiRTOC CD4/CD8 FACS plots for each experiment. Collated FACS plots of CD4 and CD8 T cell profiles of each RTOC condition for each of the three independent biological experiments. Technical replicates here refer to separate animals grafted with the same condition on the same surgery date.

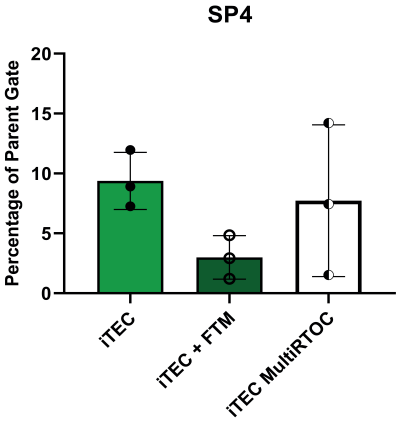
Condition		CD45$^+$ cells	DN	DP	SP4	SP8	CD3ε$^+$TCRβ$^+$ T cells
iTEC	Rep A	5.21	64.14	21.77	7.26	6.83	13.02
	Rep B	2.12	58.80	21.26	11.96	7.97	18.60
	Rep C	1.09	79.21	0.99	8.91	10.89	19.80
	Mean	2.81 ±2.14	67.38 ±10.58	14.67 ±11.85	9.38 ±2.38	8.56 ±2.09	17.14 ±3.62
iTEC + FTM	Rep A	75.31	4.58	92.16	2.93	0.33	3.39
	Rep B	80.85	5.37	86.64	4.84	3.15	5.67
	Rep C	9.18	4.20	93.54	1.20	1.07	2.66
	Mean	55.11 ±39.88	4.72 ±0.60	90.78 ±3.65	2.99 ±1.82	1.52 ±1.46	3.91 ±1.57
iTEC MultiRTOC	Rep A_Tech1	6.60	66.29	2.24	21.86	9.61	18.04
	Rep A_Tech2	56.85	23.28	64.86	6.55	5.31	5.31
	Rep A_Mean	31.73	44.79	33.55	14.21	7.46	11.68
	Rep B_Tech1	40.17	33.67	47.25	10.46	8.62	8.42
	Rep B_Tech2	58.51	77.61	14.34	4.42	3.64	3.04
	Rep B_Mean	49.34	55.64	30.80	7.44	6.13	5.73
	Rep C_Tech1	93.36	3.64	94.97	0.54	0.86	0.67
	Rep C_Tech2	68.20	37.76	56.04	2.52	3.67	1.90
	Rep C_Mean	80.78	20.70	75.51	1.53	2.27	1.29
	Mean	53.95 ±29.04	40.38 ±27.39	46.62 ±33.97	7.73 ±7.72	5.29 ±3.31	6.23 ±6.40

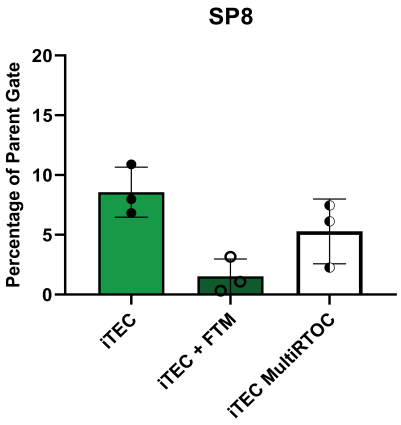
Table 4.1.2. Cell percentages of parent gate for T cell populations recovered in iTEC-MultiRTOC grafts. Detailed account of cell percentages of populations for three biological replicates per condition with mean ± SD. Rep, replicate.

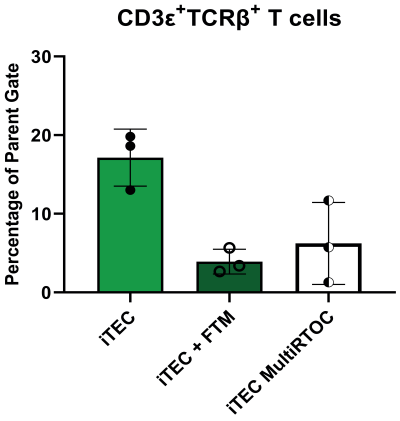

Live CD45+ cells
Percentage of Parent Gate
100
90
80
70
60
50
40
30
20
10
0
iTEC
iTEC + FTM
iTEC MultiRTOC


DN
Percentage of Parent Gate
100
90
80
70
60
50
40
30
20
10
0
iTEC
iTEC + FTM
iTEC MultiRTOC


DP
Percentage of Parent Gate
100
90
80
70
60
50
40
30
20
10
0
iTEC
iTEC + FTM
iTEC MultiRTOC


SP4
Percentage of Parent Gate
20
15
10
5
0
iTEC
iTEC + FTM
iTEC MultiRTOC


SP8
Percentage of Parent Gate
20
15
10
5
0
iTEC
iTEC + FTM
iTEC MultiRTOC


CD3ε+TCRβ+ T cells
Percentage of Parent Gate
30
20
10
0
iTEC
iTEC + FTM
iTEC MultiRTOC

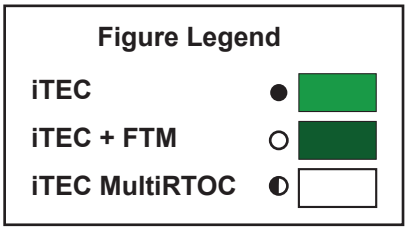


Figure 4.1.13. Cell percentages of thymocytes present in grafts 2 weeks *in vivo* in the presence or absence of FTM in single RTOC and MultiRTOC. Each datapoint represents one biological replicate. For the MultiRTOC condition each datapoint is the mean of two technical replicates. CD3ε^{+}TCRβ^{+} T cells, DN (CD4^{-}CD8^{-}), DP (CD4^{+}CD8^{+}), SP4(CD4^{+}), SP8 (CD8^{+}). DP percentages are indicative of success rate of undergoing thymopoiesis in the recovered grafts. Graphs display mean+SD. iTEC (200,000 iTEC + 100,000 WT MEFs + 100,000 DNs), iTEC + FTM (200,000 iTEC + 100,000 WT MEFs + 100,000 DNs + 50,000 FTM), iTEC MultiRTOC 3 x (200,000 iTEC + 100,000 WT MEFs + 100,000 DNs). N=3 independent biological experiments.

4.1.D. FTM is not required for iTEC-RTOC patterning

The data shown in section 4.1.B establish that RTOC containing iTEC+FTM are in some cases able to self-organize into cortical and medullary regions upon transplantation, although whether or not this occurs is variable. However, they did not establish whether self-organisation can occur in the absence of FTM. Therefore, I examined iTEC-MultiRTOC grafts recovered four weeks post transplantation for evidence of cortex:medulla organisation (data were not collected for grafts at two weeks post transplantation). This analysis was only carried out for one biologically independent experiment, due to limitations of tissue and mice.

Two recovered grafts were examined from separate animals transplanted on the same surgical date, with iTEC established from the same batch of MEFs. Brightfield images of recovered kidneys and H&E staining of recovered graft sections are shown in Figure 4.1.14. Organisation into cortical (hematoxylin dense regions; purple) and medullary (eosin dense regions; pink) regions, the presence of vast numbers of thymocytes (haematoxylin dense staining; purple) and lobule formation, are evident in the sections from Rep A but not Rep B (Figure 4.1.14.B), indicating that formation of cortical and medullary regions can occur but is variable between grafts.

To further confirm the organisation of the Rep A graft into distinct cortical and medullary regions, immunohistochemical analysis for K8, UEA1 and CD45 staining was carried out on cryosections of the recovered graft tissue (Figure 4.1.15). In agreement with the H&E results (Figure 4.1.14.B), the Rep A graft displayed distinct UEA1^{+} medullary regions and dense packing with CD45^{+} haematopoietic cells. K8^{+} cells formed a reticular structure resembling that in the RFTOC control. Distinct medullary regions are outlined by white dotted lines. The K8 reticular structure was not as distinct in the Rep B iTEC-MultiRTOC graft, and UEA1^{+} cells did not appear to form distinct regions. Lastly, CD45 staining within that graft was not as dense as would be expected in a successful graft, indicating failure of thymocyte proliferation.

Markers of functional cTEC (β5t^{+}, MHCII^{lo}) and mTEC (AIRE^{+}, MHCII^{hi}) were also examined to further characterize the organised graft. Rep A displayed a distinct region expressing high levels of MHCII (MHCII^{hi}), characteristic of thymic medulla, with the Rep B graft expressing low levels of MHCII (MHCII^{lo}) (Figure 4.1.16). MHCII^{lo} regions colocalize with β5t, consistent with thymic cortex. The pattern of β5t

and MHCII expression in the recovered iTEC-MultiRTOC was identical to that observed in transplanted whole reaggregated E15.5 thymic lobes (RFTOC).

Expression of the autoimmune regulator, AIRE, essential for the induction of self-tolerance was also analysed. As shown in Figure 4.1.17. the recovered iTEC-MultiRTOC contained a distinct $K14^+$ area that also contained $UEA1^+$ iTEC. Strikingly, this region also contained iTEC that expressed AIRE, resembling the structure of the positive control shown in the panel below.

To conclude, immunohistochemical analysis of the recovered iTEC-MultiRTOC establish that iTEC can recapitulate native thymus architecture in the absence of FTM. However, of note is that despite the two grafts recovered (Rep A and Rep B) being derived from the same batch of iTEC, having the same cellular inputs and being transplanted on the same date, they did not both establish cortical and medullary regions. This suggests that there is an unknown factor in the experiments other than input cell types (diversity within iTEC, transplantation technique, donor, vascularisation rate or rare cell types within FTM, DN or iTEC that occur at a frequency lower than 1-2 per number of cells included). Additional experiments need to be carried out to determine the proportion of grafts that do establish cortical and medullary regions but were out with the scope of this PhD due to time limitation and tissue availability.

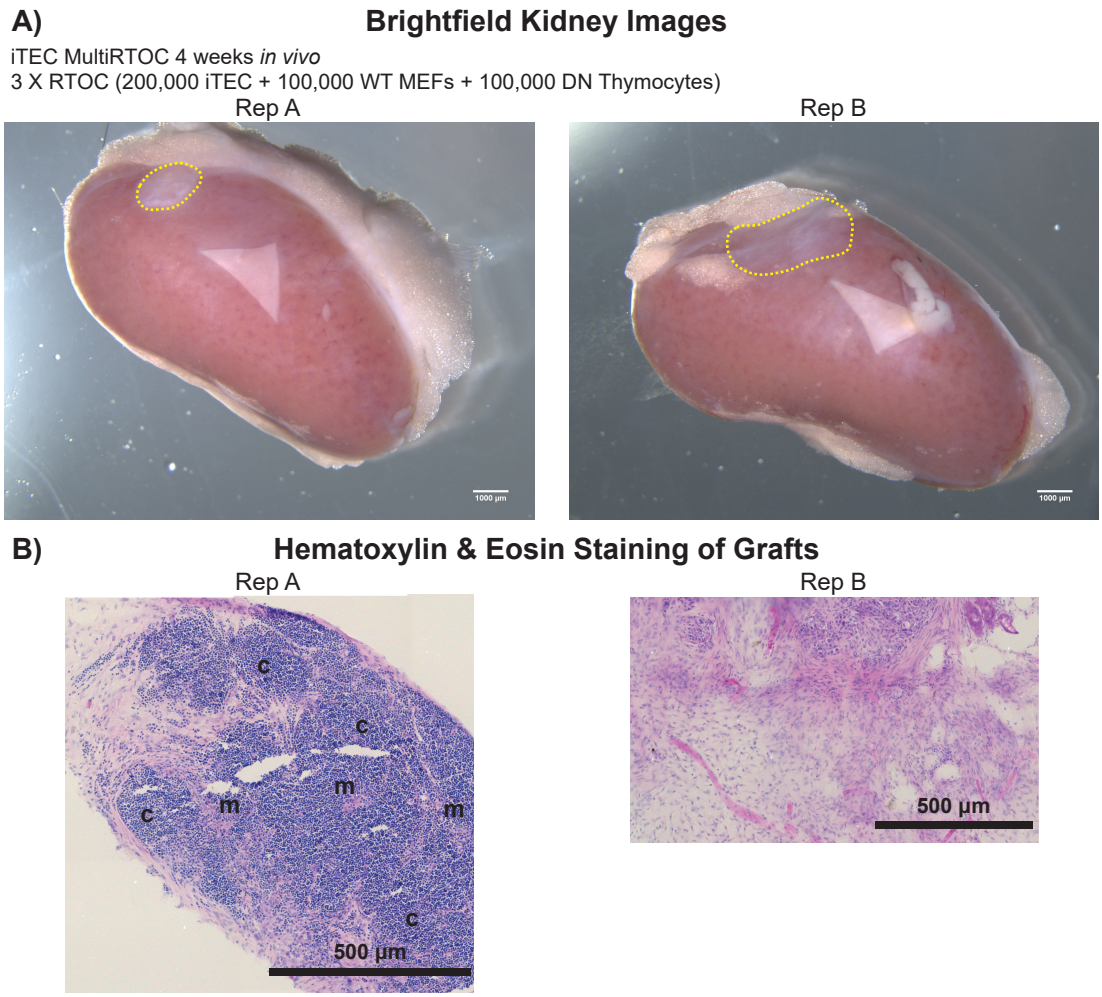


Figure 4.1.14. Histology of recovered iTEC-MultiRTOC grafts 4 weeks *in vivo*. A) Brightfield images of kidneys used for iTEC-MultiRTOC 3 x (200,000 iTEC + 100,000 WT MEFs+ 100,000 DN Thymocytes). Both recovered grafts displayed were performed with the same batch of iTEC and grafted on the same day. Grafts are outlined in yellow lines. **B)** Haematoxylin and eosin (H&E) staining of sectioned recovered grafts. Demarkation between cortical (c) and medullary (m) areas. Cortical areas are classified with dense hematoxylin staining (purple) as an indication of an abundance of thymocytes. Medullary areas are classified with dense eosin staining (pink) as an indication of less thymocytes present. Rep A shows organisation into cortical and medullary regions with an abundance of thymocytes present in the graft. Rep B failed to self-organise and support proliferation of thymocytes. N=1 independent biological experiment.

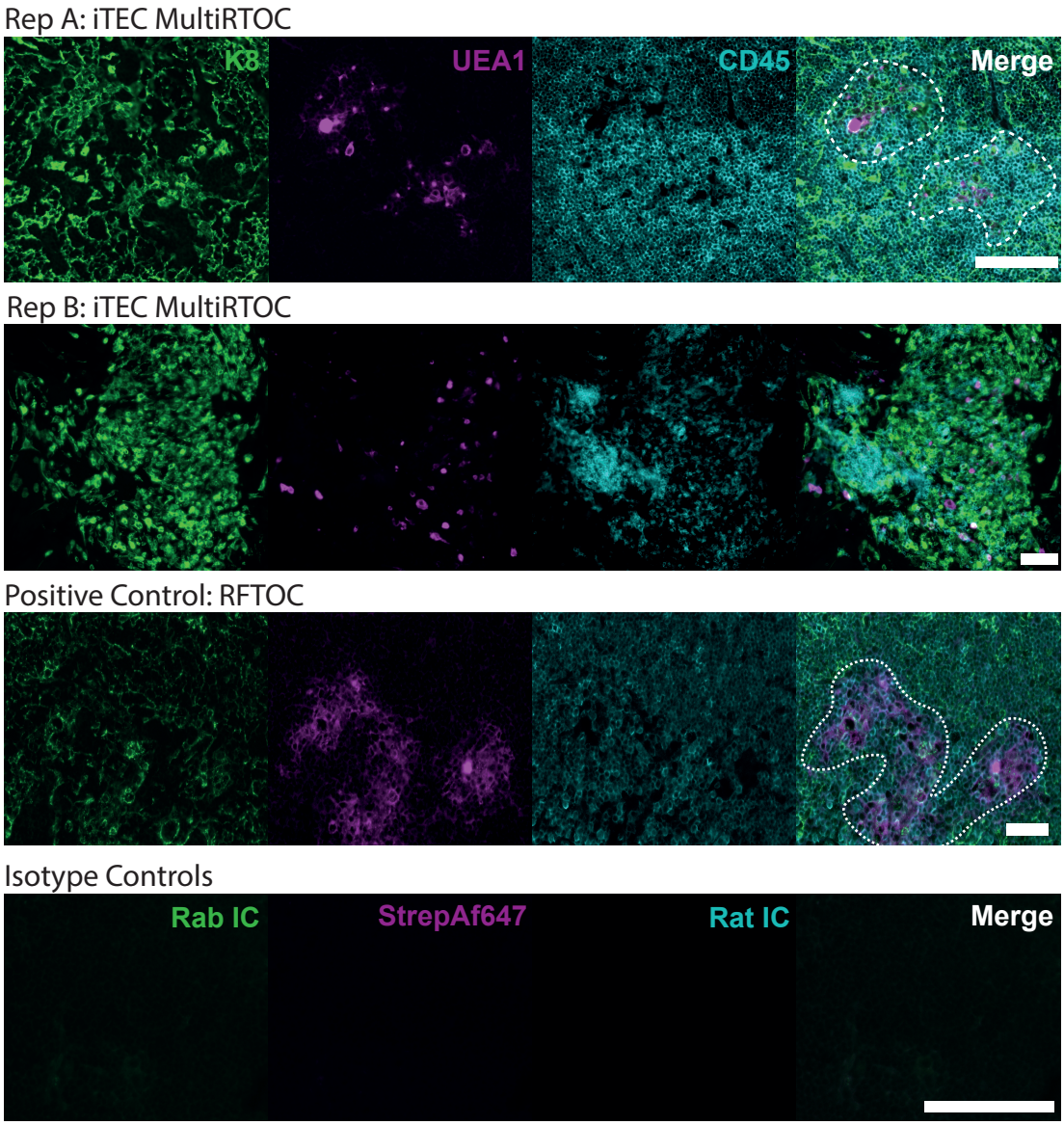
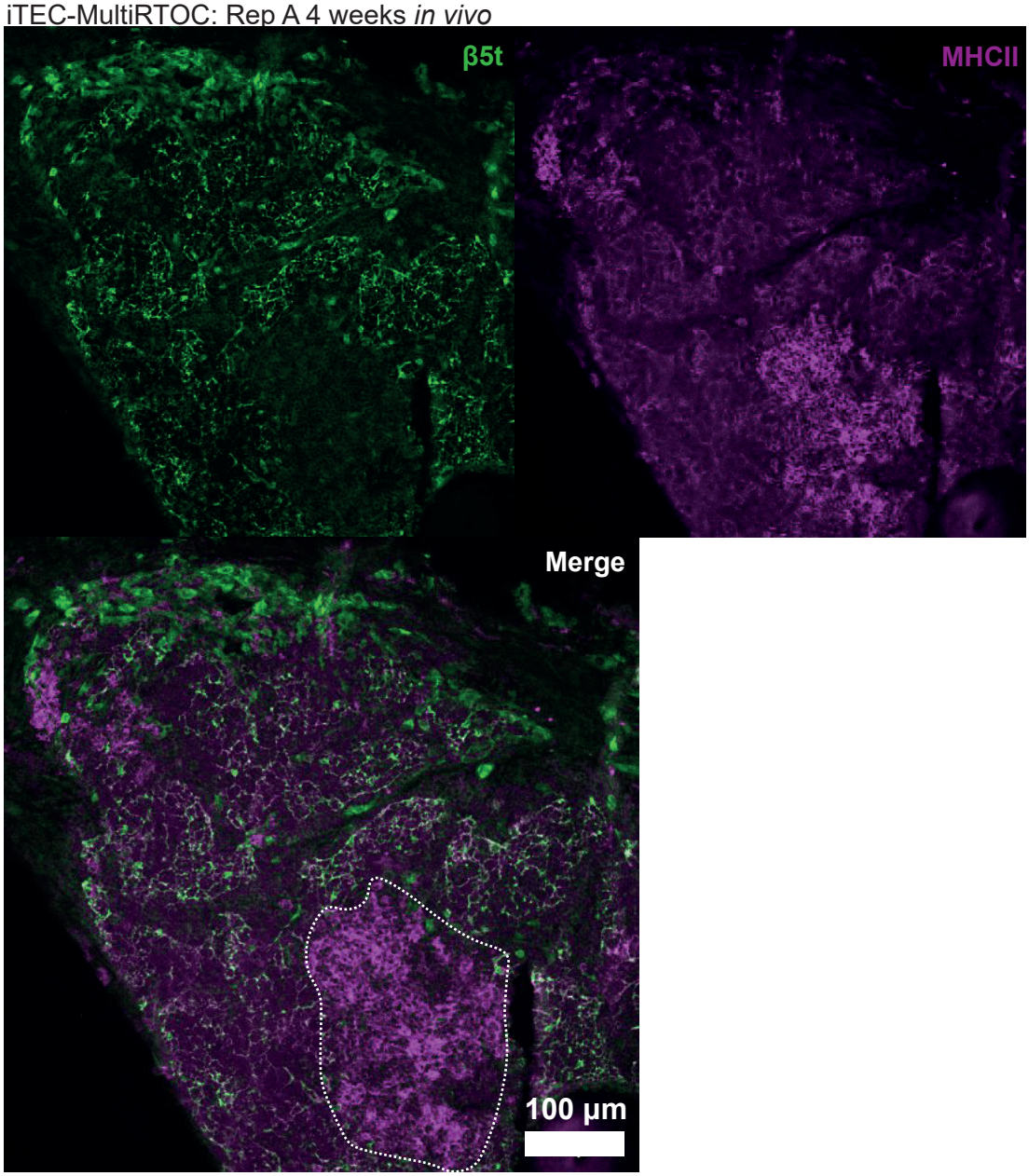


Figure 4.1.15. Immunohistochemistry staining of iTEC-MultiRTOC recovered grafts 4 weeks *in vivo*. Grafts were stained with cytokeratin 8-K8 (green) for cortical regions, UEA1 (magenta) for medullary regions and CD45 (cyan) for cells of haematopoietic origin. For each condition, representative regions of interest were selected to showcase whether organisation into distinct cortical and medullary regions had formed in the graft. Positive controls include recovered RFTOC at 2 weeks *in vivo*. The negative control used was stained for isotypes of the primary antibody, where possible, and followed by secondary antibody staining. Images for Rep A and Rep B of iTEC show variation between technical replicates on outcome of histology. Scale bar 100 µm. iTEC-MultiRTOC: 3 x (200,000 iTEC + 100,000 WT MEFs + 100,000 DNs).

iTEC-MultiRTOC: Rep A 4 weeks *in vivo*



Positive Control: RFTOC

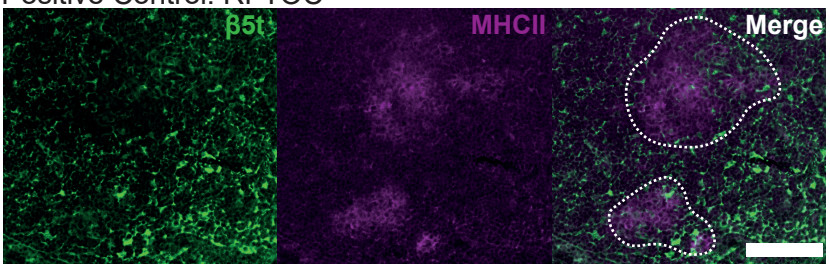
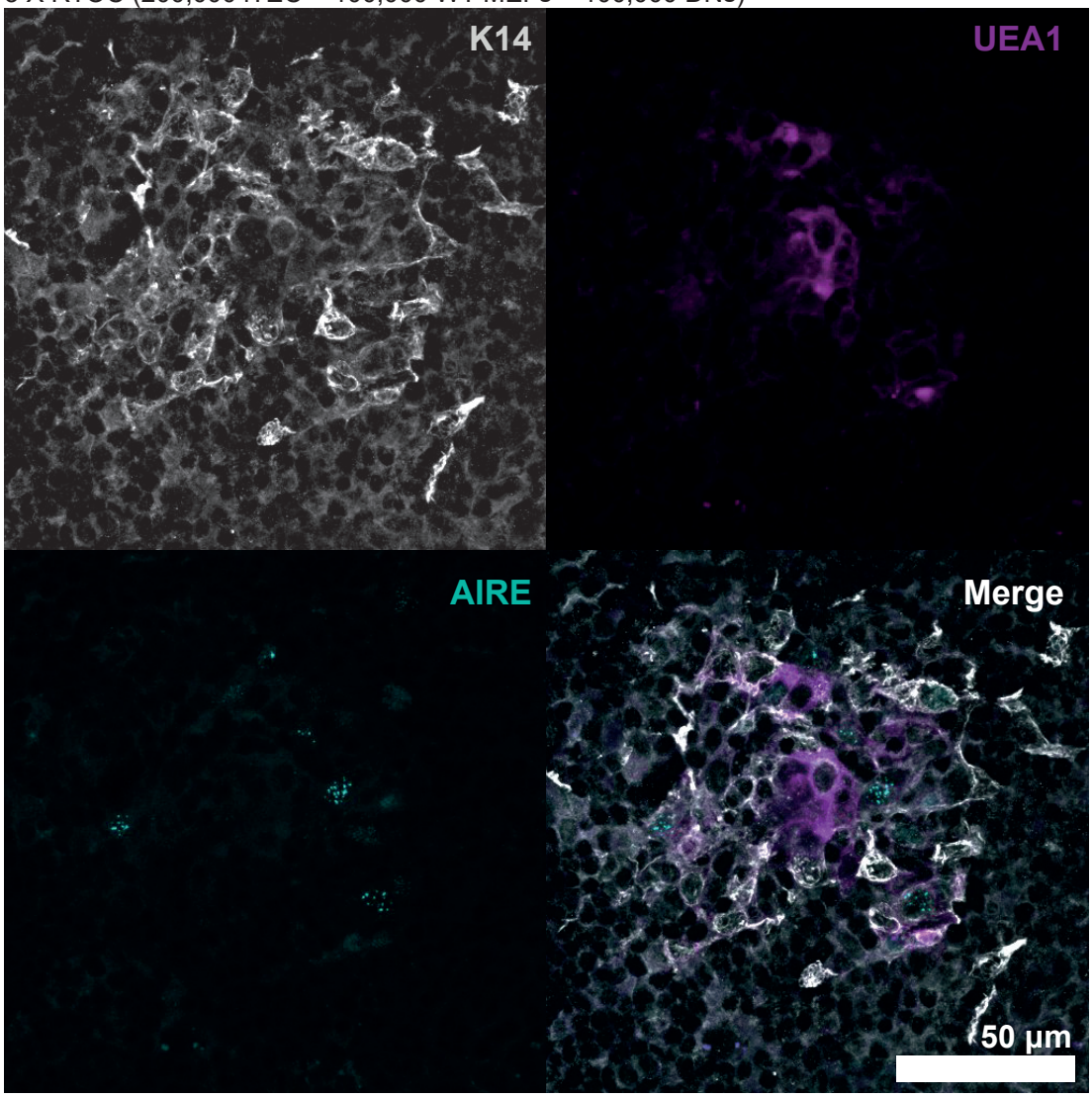


Figure 4.1.16. Immunohistochemistry of additional markers in successfuly organised recovered iTEC-MultiRTOC graft 4 weeks *in vivo*. Sections were stained with β5t (green) for cortical regions and MHCII (magenta) for cortical regions ($MHCII^{lo}$), medullary regions ($MHCII^{hi}$). A large medullary regions is outlined in a white dotted line. 100 µm scale bar. Positive control is a recovered RFTOC 2 weeks *in vivo*.

iTEC MultiRTOC: Rep A 4 weeks *in vivo*
3 X RTOC (200,000 iTEC + 100,000 WT MEFs + 100,000 DNs)



Positive Control: RFTOC

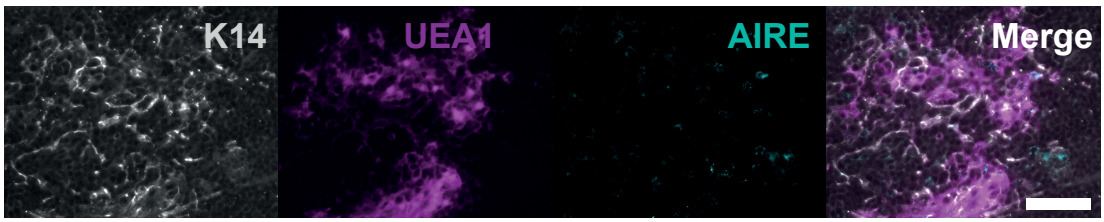


Figure 4.1.17. Medullary region histological examination of successfully organised recovered iTEC-MultiRTOC graft 4 weeks *in vivo*. The grafts was stained with cytokeratin 14-K14 (grey) and UEA1(magenta) for medullary regions and autoimmune regulatory element-AIRE (cyan). Note spotty nature in staining of AIRE as it is a transcription factor and therefore localised in the nucleus of the cells. The graft transplanted under the kidney capsule of a syngeneic C57BL/6J mouse consisted of the iTEC-MultiRTOC condition 3 x (200,000 iTEC+ 100,000 WT MEFs + 100,000 DNs). In the absence of FTM the iTEC-MultiRTOC was able to self-organise *in vivo* and form distinct medullary islands that co-express medullary phenotypical and functional markers such as K14, UEA1 and AIRE respectively. 50 µm scale bar. Positive control is a recovered RFTOC 2 weeks *in vivo*.

4.2. Smart-Seq2 FTM vs WT MEFs: Identification of factors produced by FTM but not WT MEF that may affect TEC

Although Anderson and colleagues (1993) showed that the 3T3 fibroblast cell line was able to replace FTM in native RFTOC, primary WT MEFs established on a syngeneic background to iTEC were not able to recapitulate this effect. As FTM (neural crest derived) and WT MEFs are of different origins, different gene expression profiles are expected. Therefore, we hypothesised that factors expressed by FTM but not MEFs were responsible for the effects shown above.

To test this, I performed Smart-Seq2 RNA-sequencing of bulk E15.5 FTM and MEF populations. Three independent experiments were carried out for the collection of FTM and WT MEF samples to be sent for sequencing. For each dataset, three biological replicates with three technical replicates each was collected resulting in a total of 9 samples per dataset. 100 cells of each cell type were sorted into Smart-Seq2 lysis buffer using a BD FACSAria^{TM} cell sorter. Representative FACS plots and samples collected for RNA-Sequencing can be viewed in Figure 4.2.1.

The resulting datasets were used in differential gene expression analysis, to obtain a list of cell surface ligands and secreted growth factors and cytokines expressed by FTM but not MEFs, and from this list, genes whose products interact with receptors on native E14.5 TEC were identified (a dataset of E14.5 PLET1^{+} and PLET1^{-} TEC was already available from the Blackburn lab as described in (Liu *et al*., 2020)). This bioinformatics analysis was carried out by Dr Paul Rouse (Blackburn lab), as outlined below.

We hypothesised that supplementation of iTEC-RTOC with one or more of the candidate factors identified would positively impact iTECs ability to support thymopoiesis in the absence of FTM.

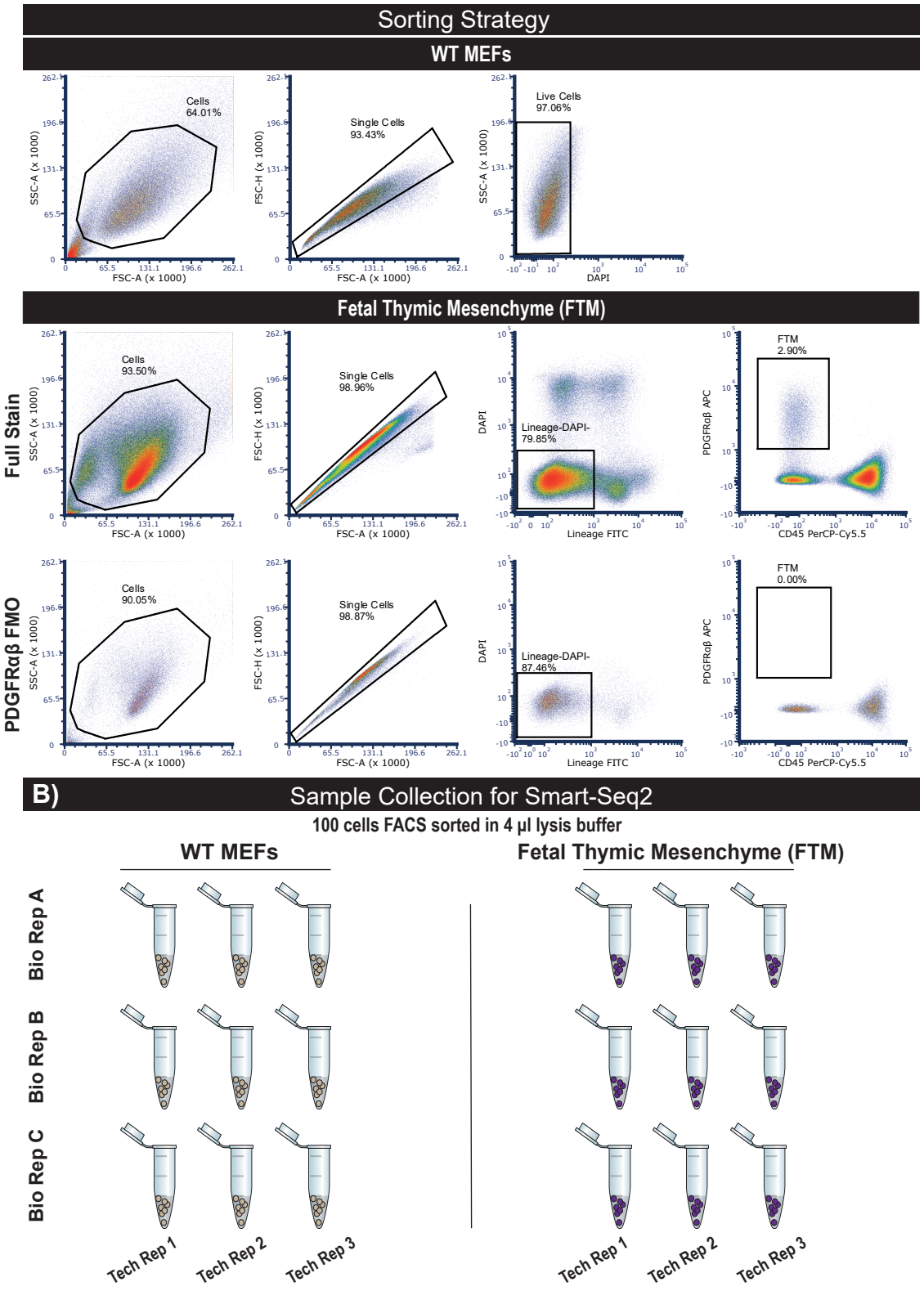


Figure 4.2.1. Sample collection for Smart-Seq2 RNA-Sequencing of WT MEFs vs FTM. A) FACS sorting strategy for collection of WT MEFs (DAPI-) and FTM (Lin-PDGFRαβ+) . Lineage (CD3, CD4, CD8, CD11b, CD11c, B220, Gr-1, NK1.1, TER-119). **B)** 100 cells were sorted into 10 µl lysis buffer. Three technical replicates (Tech Rep) were collected for each experiment. N=3 independent biological experiments (Bio Rep).

4.2.A. FTM and WT MEFs have distinct transcriptional profiles

First, principal component analysis (PCA) was carried out using the most variable 100,000 genes on the FTM and WT MEFs datasets. These separated into three distinct groups based on PCA1 and PCA2 (Figure 4.2.2). The FTM group formed a tight cluster. Biological replicate 1 and 2 of WT MEFs also formed a tight cluster. Biological replicate 3 of WT MEFs, however, formed a distinct cluster which was close in distance on PC1 but exhibited large variation on PC2. As WT MEFs are primary cultures, batch to batch variability in their expression profiles is expected. Of note is that each biological replicate was established from a separate embryo, which may have had slight differences in developmental timings. Also of note is that batch-to-batch variation in MEFs may be a contributing factor to iTEC variability.

A breakdown of PC1 and PC2, and the top 10% of most variable genes found in those contributing to the clustering of the samples, are depicted in Figure 4.2.3.A and listed below:

PC1 (*CT010467.1, Acta2, Ccn3, Mfap4, Rrm2, Tm4sf1, Tubb6, Apod, Kpna2, Gm10184, Gm10259, Rrm1, Ccnb1, Mfap2, Agtr2, Dcn, Enpp2, Lum, Gdf10*, *Itm2a, Mest*).

PC2 (*Kpna2, Ccnb1, Gm10184, Tub66, Rrm2, Gm10259, Rrm2, Gm10259, Rrm1, Tm4sf1, Acta2, Gdf10, Enpp2, Mest, Itm2a, Agtr2, Lum, CT010467.1, Dcn, Mfap2, Apod, Ccn3, Mfap4*).

Contribution of other principal components can be seen in the SceePlot in Figure 4.2.3.B.

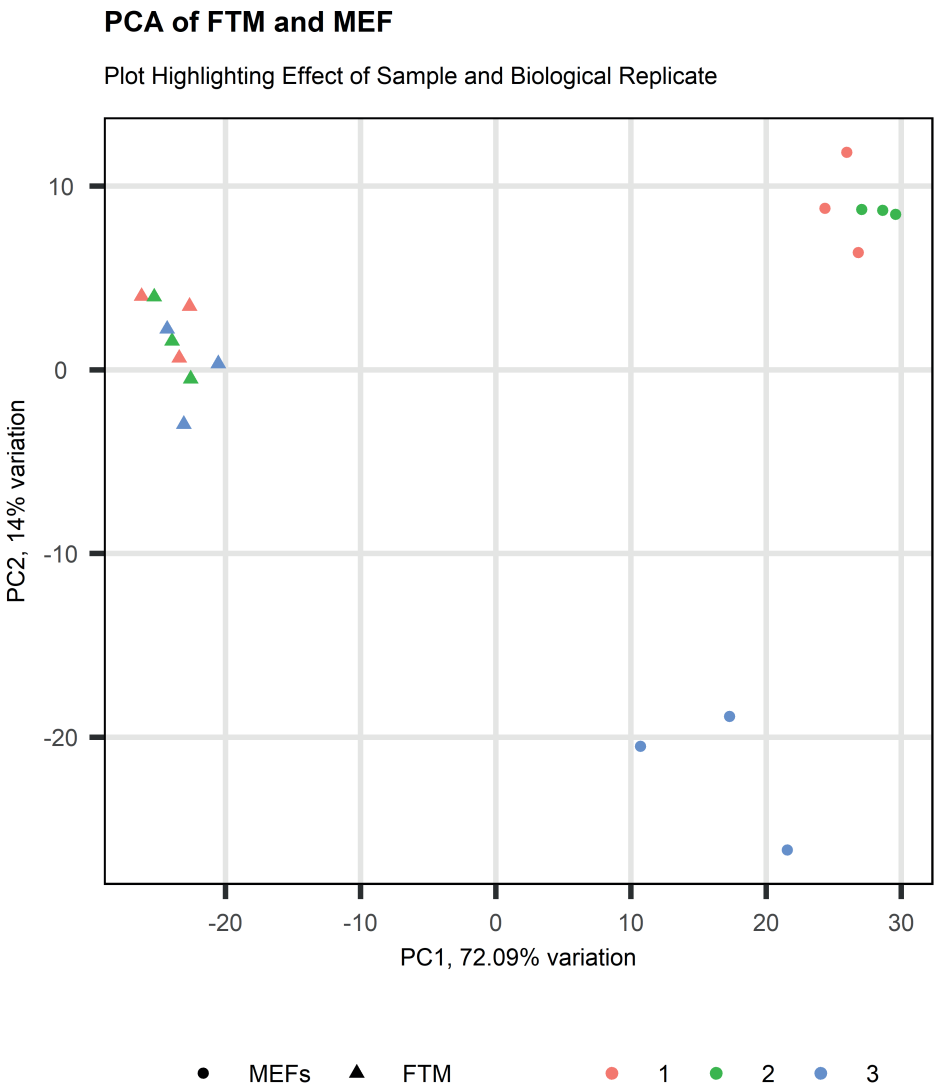


Figure 4.2.2. Principal component analysis of FTM and MEF dataset. The PCA plot shows sample clustering with MEFs represented with circles and FTM with triangles. The three biological replicates are distinguished with red, green and blue and each biological replicate is further divided into three technical replicates. PC1 (x-axis) accounts for 72.09% of sample variance and PC2 (y-axis) for 14%.

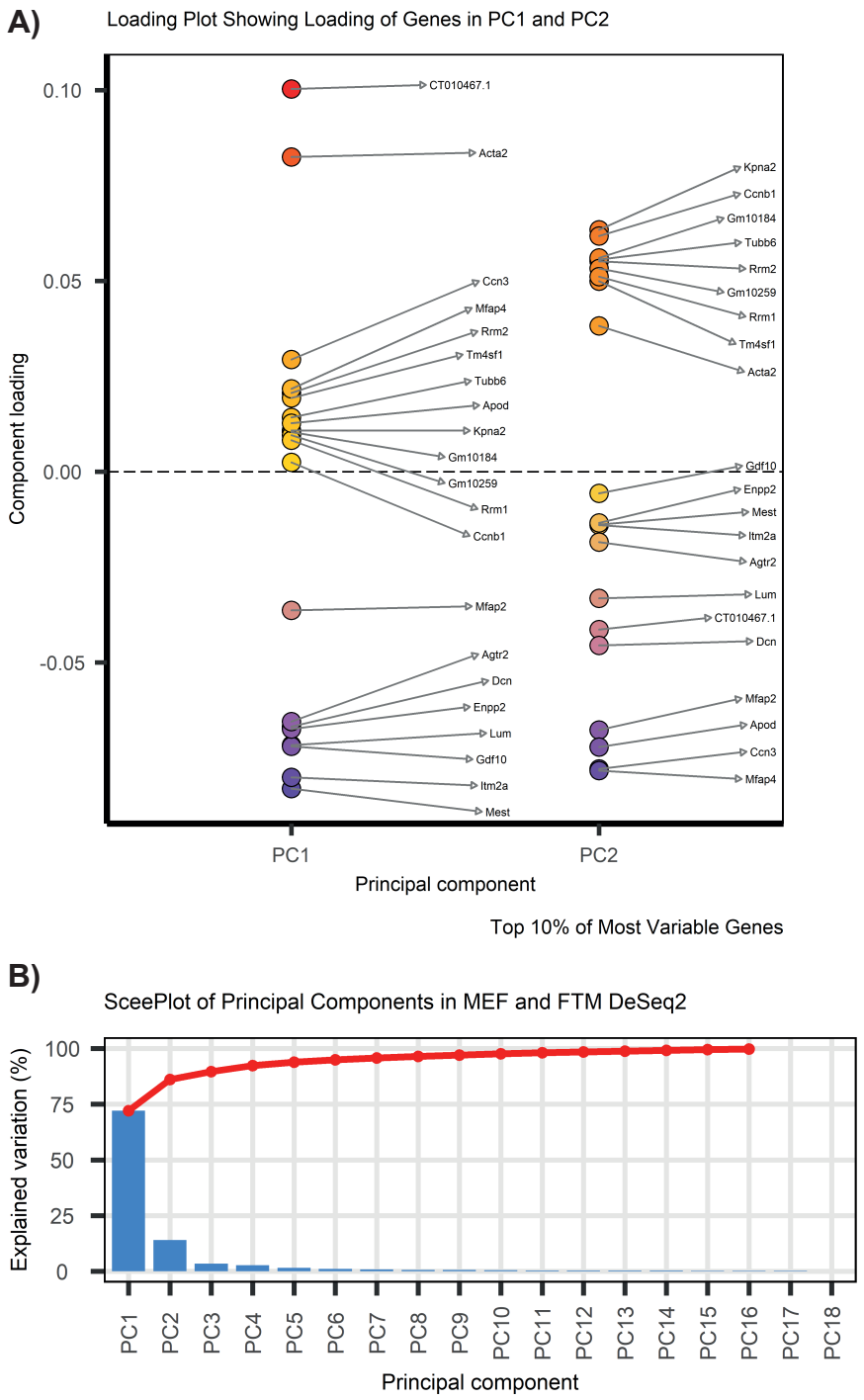


Figure 4.2.3. Principal components PC1 and PC2. A) Loading plot showing loading of genes in PC1 and PC2 for MEF and FTM datasets. Top 10% of most variable genes associated with PC1 and PC2. **B)** SceePlot of Principal components in MEF and FTM DESeq2. Breakdown of percentage of contribution of each principal component to the variation found in MEF and FTM samples.

4.2.B. FTM, MEF and E14.5 TEC all have distinct transcriptional profiles

A further principal component analysis was performed that included the E14.5 PLET1^{+}EPCAM^{+} and E14.5 PLET1^{-}EPCAM^{+} TEC datasets (Figure 4.2.4). The addition of these datasets resulted in the three distinct clusters. PLET1^{+} (progenitor like TEC) and PLET1^{-} (differentiating TEC) E14.5 TEC were both included in the analysis, since iTEC are likely to be heterogeneous and can generate differentiated TEC subtypes. One group comprised of all the TEC samples, the second group of all the FTM samples and the third of all the WT MEF samples, indicating the distinct transcriptional profiles of each cell population. The variability of the third biological replicate of WT MEFs was not as evident in the analysis, because of the strong differences between the three broad cell types.

Bi-plots exhibiting overlaid expression of the key genes comprising each principal component clustered as would be expected (Figure 4.2.5). High levels of *Foxn1* expression (red) can be seen in TEC but not in WT MEF or FTM. *Pdgfrα*, a mesenchymal marker used to isolate FTM was highly expressed on FTM but not WT MEFs or TEC. Interestingly, expression of *Dll4* was found in FTM and TEC (although higher in TEC than FTM) but not WT MEFs. Lastly, chemokine ligand 7 (*Ccl7*) was expressed most highly in WT MEFs. CCL7 is a lymphocyte migration marker and is known to be expressed by a variety of cells including fibroblasts and epithelial cells (Liu *et al*., 2018).

Hierarchical clustering based on sample distances was performed based on overall gene expression and normalised using the DESeq2 Bioconductor package. The expression heatmap of sample-to-sample distances between the datasets revealed that all E14.5 TEC clustered together, independently of the PLET1^{+} or PLET1^{-} phenotype, all FTM clustered together, and all WT MEFs clustered together (Figure 4.2.6). Corresponding technical and biological replicates do not cluster together, with the exception of MEF Bio Rep 3, indicating the absence of batch effects due to day of experiment or embryo from which cells were obtained.

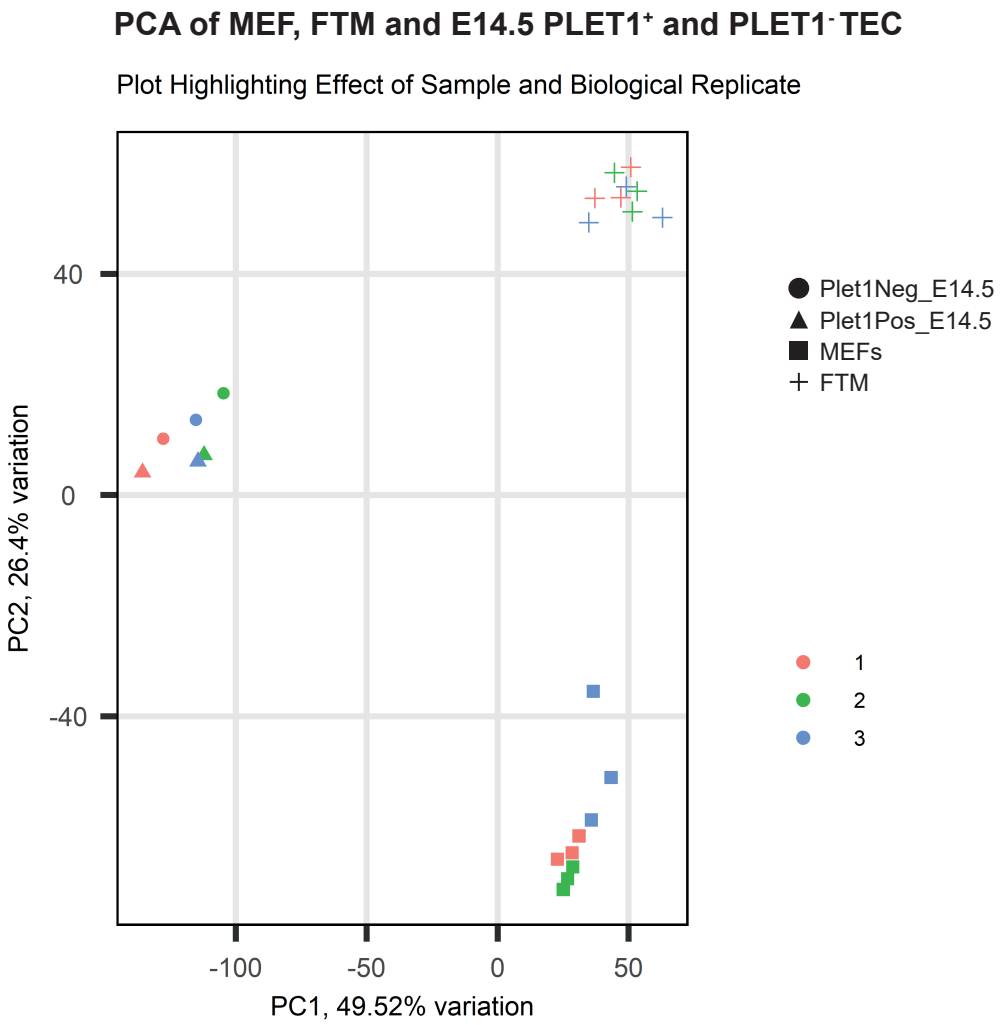


Figure 4.2.4. Principal component analysis of MEF, FTM and E14.5 TEC datasets. The PCA plot shows sample clustering with MEFs represented with squares, FTM with crosses, PLET1^{-} E14.5 TEC (Plet1Neg_E14.5) with circles and PLET1^{+} E14.5 TEC (Plet1Pos_E14.5) with triangles. The three biological replicates are distinguished with red, green and blue. Technical replicates are available for the MEF and FTM datasets. PC1 (x-axis) accounts for 49.52% of sample variance and PC2 (y-axis) for 26.4%.

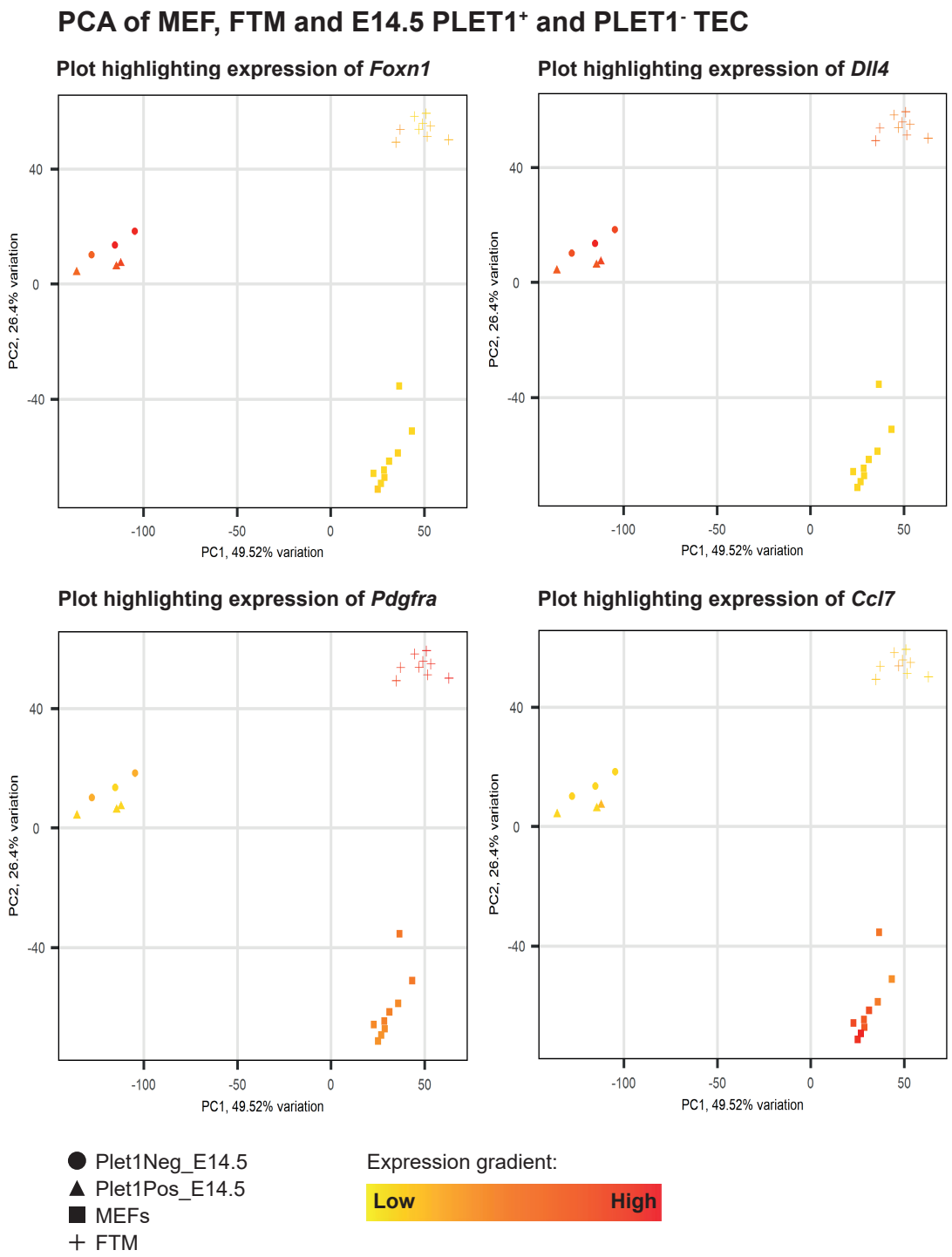


Figure 4.2.5. Bi-plots of key gene expression on PCA plot of FTM, MEF and E14.5 TEC datasets. *Foxn1* (master regulator gene of thymic epithelial cells), *Dll4* (Delta like ligand 4, downstream target of FOXN1 and the non-redundant Notch ligand required for T cell lineage commitment), *Pdgfrα* (marker of mesenchymal cells in the thymus), *Ccl7* (lymphocyte migration). Expression levels of *Foxn1, Dll4, Pdgfra* and *Ccl7* were overlayed onto the PCA plot of FTM, MEF and E14.5 TEC dataset.

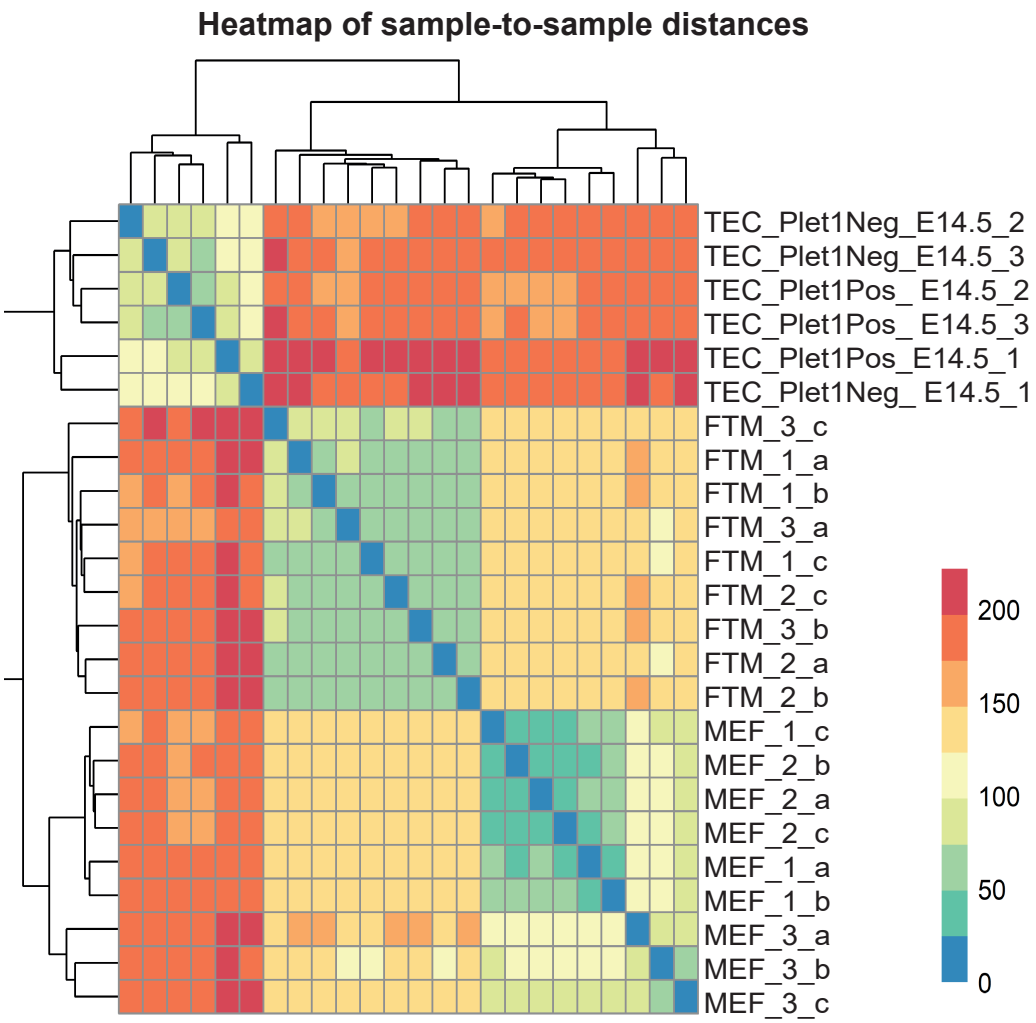


Figure 4.2.6. Expression heatmap of sample-to-sample distances between FTM, MEF and E14.5 TEC datasets. Hierarchical clustering based on sample distances was performed based on overall gene expression using DESeq2. Blue indicates similar expression and red indicates largely varied expression (colour key in arbitrary units). E14.5 TEC cluster together independent of PLET1 positive or negative nature of the cells. All three cell type samples cluster independent to each other and show complete separation from the others displaying the difference in gene expression profile between the three cell types. Within each cell type technical and biological replicates don't cluster together (with the exception of MEF Bio Rep 3) displaying the lack of batch effects present.

4.2.C. Ligand-receptor analysis: FTM-TEC, WT MEF-TEC

A table was generated containing the number of aligned transcripts (using the program STAR) for each gene for all samples (TEC, FTM, MEF). These counts were normalised using the DESeq2 Bioconductor package. Normalised FTM gene counts were multiplied with TEC counts and the same was done for MEF and TEC counts generating two outputs: FTM x TEC normalised counts and MEF x TEC normalised counts. Cell-to-cell interactions between FTM-TEC and MEF-TEC were determined by filtering data to only contain FTM or MEF ligands multiplied with their corresponding TEC receptors, as described by Ramilowski *et al*., 2015. Ligand-receptor pairs for FTM-TEC and the corresponding MEF-TEC pairs were compared and a Wilcoxon test was used to determine pairs that were significantly different between the two with a False Discovery Rate of < 0.1.

4.2.D. Candidate selection and experimental design

In total, 445 ligand-receptor pairs were significantly upregulated (based on the Wilcoxon test) in FTM-TEC compared to WT MEF-TEC and 528 pairs in MEF-TEC over FTM-TEC (Appendix Table 8.1). These numbers reflect that in many instances, a ligand could bind to more than one receptor.

Candidate pairs in which the ligand was expressed more highly in FTM than MEF were selected for functional testing of the hypothesis that FTM provides a factor or factors, that are not expressed in MEFs and that enhance iTEC support of thymopoiesis in the RTOC system. Of these candidates only ones commercially available through Peprotech® were taken forward for testing, to ensure standardisation of factor production. The selected ligands and their corresponding receptors are shown in Table 4.2.1. The twenty-five candidates were taken forward and an *in vitro* screen was designed that tested whether their supplementation in culture media in the iTEC-RTOC system could replace the effect FTM displayed (Table 4.2.2).

Due to time restrictions and tissue availability, these experiments were selective, as development of a high throughput factor screen was not an option. Candidate growth factors were initially grouped into pools for testing and, due to the variability of the iTEC RTOC system, a minimum of three technical replicates were performed per condition. The groups were as follows: no candidates, all candidates, group 1, group 2, group 3. Candidates were randomly allocated into groups using a random number generator to avoid bias. Another method which was not explored could be to

group candidates based on receptor usage or known synergy. The groups, RTOC conditions and their corresponding media supplementation are shown in Figure 4.2.7. DMEM/F12 was used as the base medium and was supplemented with 2% rather than 10% fetal calf serum (FCS) to reduce the impact of factors found in serum. RTOC were cultured for fourteen days to allow thymopoiesis to take place and then processed for flow cytometry analysis. All candidate factors were used at a concentration of 5ng/ml. Lymphoid-primed multipotent progenitors (LMPPs: $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}Flt3^{+}$) were used rather than E15.5 DNs, as they have good proliferative capacity and are routinely used in the *in vitro* iTEC co-cultures by the Blackburn lab (Paul Rouse, unpublished).

iTEC, LMPPs, E15.5 TEC and FTM were all collected using a BD FACSAria™ cell sorter. Representative FACS plots for each cell type are shown in Figure 4.2.8.A. RTOC were cultured at the medium-gas interface on isopore membrane filters (hydrophilic polycarbonate membrane, 0.8 μm pore size, Merck, ATTP01300) for fourteen days. 50% medium changes were implemented every second day, with the corresponding 2X candidate medium condition. At the experimental endpoint, RTOC were collected digested for 5 minutes with TrypLE Express (which was shown not to cleave the surface markers assayed in experiment), mechanically dissociated with a 25G needle and syringe and pooled with the medium obtained from the well it had been cultured in to ensure that any thymocytes already released from the RTOC were included in the analysis (Figure 4.2.8.B). RTOC conditions used included iTEC RTOC (600,000 iTEC + 100,000 WT MEFs + 1,000 LMPPs) with either no candidate factors, all candidates, or group 1, group 2 or group 3 candidates supplemented into the media. iTEC + FTM (600,000 iTEC + 50,000 FTM + 100,000 WT MEFs + 1,000 LMPPs) with no candidate factors added, and native E15.5 TEC RTOC (300,000 E15.5 TEC + 100,000 WT MEFs + 1,000 LMPPs) as positive controls. A lower number of native TEC was used due to their limited availability in this experiment. This experiment was only carried out once and therefore the resulting data are preliminary.

Ligand	Receptor	Adjusted_p_value (FDR)	Log2_FC_MEF_minus_FTM
HGF	CD44	4.60E-18	-0.42
	SDC1	4.60E-18	-0.42
	MET	4.60E-18	-0.42
	ST14	4.77E-18	-0.42
IL15	IL15RA	4.85E-18	-0.31
	IL2RA	1.04E-17	-0.31
	IL2RG	3.42E-16	-0.31
TSLP	IL7R	3.74E-15	-0.12
IGF1	INSR	6.27E-16	-0.38
	IGF1R	2.51E-17	-0.38
	INSR	6.27E-16	-0.38
	IGF1R	2.51E-17	-0.38
BMP2	ACVR1	0.028125511	-0.05
	ACVR2A	0.028532465	-0.05
	ACVR2B	0.017982122	-0.05
	BMPR1A	0.020315644	-0.05
	BMPR2	0.034492955	-0.05
PGF	FLT1	4.60E-18	-0.30
	NRP1	3.22E-14	-0.30
	NRP2	1.32E-12	-0.30
EGF	ERBB4	0.026471289	-0.04
MDK	PTPRZ1	3.64E-08	-0.13
	ALK	1.74E-09	-0.13
	GPC2	2.66E-09	-0.13
	ITGA4	1.44E-08	-0.13
	ITGA6	4.07E-09	-0.13
	ITGB1	2.92E-09	-0.13
	LRP1	1.56E-05	-0.13
	LRP2	0.001514226	-0.13
	SDC1	5.90E-09	-0.13
	SDC3	3.78E-09	-0.13
	SDC4	6.11E-09	-0.13
	PTPRB	1.80E-07	-0.13
PF4	FGFR2	4.60E-18	-0.41
	SDC2	4.60E-18	-0.41
	LDLR	4.60E-18	-0.41
	PROCR	4.60E-18	-0.41
	THBD	4.60E-18	-0.41
PTN	PLXNB2	8.66E-12	-0.19
	PTPRB	5.70E-11	-0.19
	ALK	4.21E-14	-0.19

	CDH10	6.67E-12	-0.19
	PTPRS	3.66E-12	-0.19
	PTPRZ1	3.80E-11	-0.19
	SDC1	1.07E-11	-0.19
	SDC3	1.72E-12	-0.19
VEGFA	ITGAV	6.26E-15	-0.16
	EGFR	5.86E-17	-0.16
	FLT1	4.60E-18	-0.16
	GPC1	3.15E-13	-0.16
	NRP2	2.04E-06	-0.16
	SIRPA	9.96E-18	-0.16
	TYRO3	6.54E-18	-0.16
	EPHB2	5.28E-09	-0.16
	ITGA9	5.32E-17	-0.16
	ITGB1	4.60E-18	-0.16
	KDR	4.60E-18	-0.16
	NRP1	1.30E-08	-0.16
	RET	1.08E-10	-0.16
BMP4	BMPR2	4.71E-12	-0.13
	ACVR1	1.02E-12	-0.13
	ACVR2A	2.63E-13	-0.13
	BMPR1A	7.22E-15	-0.13
	BMPR1B	4.50E-10	-0.13
TGFB2	ACVR1	1.47E-13	-0.15
	ENG	9.27E-13	-0.15
	TGFBR1	3.97E-17	-0.15
	TGFBR2	3.22E-15	-0.15
	TGFBR3	2.21E-16	-0.15
ANGPTL7	TIE1	4.60E-18	-0.39
IL16	CCR5	2.52E-13	-0.24
	CD4	3.37E-13	-0.24
	GRIN2C	2.15E-13	-0.24
	KCNA3	1.01E-12	-0.24
	KCND1	1.99E-12	-0.24
	KCNJ15	4.41E-13	-0.24
	KCNJ4	3.22E-13	-0.24
	GRIN2D	1.89E-13	-0.24
	KCND2	2.10E-13	-0.24
IGFBP4	FZD8	5.34E-13	-0.29
	LRP6	2.01E-13	-0.29
IGF2	IGF1R	2.12E-16	-0.16
	IGF2R	1.88E-12	-0.16
	INSR	7.05E-14	-0.16

IL34	CSF1R	0.029077556	-0.03
SLIT2	DCC	0.034670327	-0.02
FGF18	FGFR1	3.66E-12	-0.21
	FGFR2	3.28E-13	-0.21
	FGFR3	2.34E-12	-0.21
BMP7	BMPR1A	4.60E-18	-0.30
	ACVR2A	7.82E-18	-0.30
	BMPR1B	8.70E-17	-0.30
	ACVR2B	4.60E-18	-0.30
	ENG	2.24E-17	-0.30
	ACVR1	5.88E-18	-0.30
RARRES2	CCRL2	4.60E-18	-0.51
	GPR1	4.60E-18	-0.51
	CMKLR1	4.60E-18	-0.51
FGF10	FGFR3	4.60E-18	-0.66
	FGFR1	4.60E-18	-0.66
	FGFR2	4.60E-18	-0.66
ANGPT1	ITGA5	9.40E-12	-0.19
	TEK	2.32E-09	-0.19
	TIE1	2.63E-12	-0.19

Table 4.2.1. Ligand-Receptor pairs significantly upregulated in FTM-TEC over MEF-TEC. Ligand-receptor pairs for FTM-TEC and the corresponding MEF-TEC pairs were compared and a Wilcoxon test was used to determine pairs that were significantly different between the two with a False Discovery Rate (FDR) < 0.1. Negative value of log2_FoldChange indicates ligand-receptor pair scored higher in FTM than MEFs. 445 ligand-receptor pairs were upregulated in FTM and 528 in MEFs. Only pairs upregulated in FTM were carried forward and from this list only ligands commercially available from Peprotech were selected for candidate validation. These candidates and their corresponding receptor on TEC are displayed in this table. This list generated a selection of 25 candidates to be tested, upregulated in FTM over MEFs that act on TEC, for replacing the function of FTM in RTOC.

Abbreviation	Candidate	Species	Catalogue Number
HGF	Hepatocyte Growth Factor	Murine	315-23
IL-15	Interleukin-15	Murine	210-15
TSLP	Thymic Stromal Lymphopoietin	Human	300-62
IGF-1	Insulin-like Growth Factor-1	Murine	250-19
FGF-1	Fibroblast Growth Factor-1	Murine	450-33
BMP-2	Bone Morphogenetic Protein-2	Human/Murine/Rat	120-02
PGF	Placental Growth Factor-1	Human	AF-100-06
EGF	Epidermal Growth Factor	Murine	315-09
MK	Midkine	Murine	315-25
CXCL4 (PF4)	Platelet Factor-4	Murine	250-39
PTN	Pleiotrophin	Human	450-15
VEGF-A	Vascular Endothelial Growth Factor A	Murine	450-32
BMP-4	Bone Morphogenetic Protein-4	Murine	315-27
TGF-β2	Transforming Growth Factor β-2	Human	100-35B
ANGPTL-7	Angiopoietin-like Protein-7	Human	130-22
IL-16	Interleukin-16	Human	200-16A
IGF-2	Insulin-like Growth Factor-2	Human	100-12
IL-34	Interleukin-34	Human	200-34
SLIT2	Slit Guidance Ligand 2	Human	150-11
FGF-18	Fibroblast Growth Factor-18	Human	100-28
IGFBP4	Insulin-like Growth Factor-Binding Protein 4	Human	350-05B
BMP-7	Bone Morphogenetic Protein-7	Human	120-03P
RARRES2	Chemerin	Human	300-66
FGF-10	Fibroblast Growth Factor-10	Human	100-26
ANG-1	Angiopoietin-1	Human	130-06

Table 4.2.2. List of 25 candidates selected from ligand-receptor analysis for experimental validation. Candidates were all purchased from Peprotech®. Where recombinant murine proteins were not available the corresponding human one was selected.

Preliminary FTM Candidate Validation *in vitro* Experimental Design
Can FTM Function be Replaced by Candidates

Selected Candidate Randomised Grouping

Group 1	**Group 2**
Hepatocyte Growth Factor (HGF)	Platelet Factor-4 (CXCL4)
Interleukin-15 (IL-15)	Pleiotrophin (PTN)
Thymic Stromal Lymphopoietin (TSLP)	Vascular Endothelial Growth Factor A (VEGF-A)
Insulin-like Growth Factor-1 (IGF-1)	Bone Morphogenetic Protein-4 (BMP-4)
Fibroblast Growth Factor-1 (FGF-1)	Transforming Growth Factor β-2 (TGF-β2)
Bone Morphogenetic Protein-2 (BMP-2)	Angiopoietin-like Protein-7 (ANGPTL-7)
Placental Growth Factor-1 (PGF)	Interleukin-16 (IL-16)
Epidermal Growth Factor (EGF)	Insulin-like Growth Factor-2 (IGF-2)
Midkine (MK)	

Group 3
Interleukin-34 (IL-34)
Slit Guidance Ligand 2 (Slit2)
Fibroblast Growth Factor-18 (FGF-18)
Insulin-like Growth Factor-Binding Protein 4 (IGFBP4)
Bone Morphogenetic Protein-7 (BMP-7)
Chemerin (RARRES2)
Fibroblast Growth Factor-10 (FGF-10)
Angiopoietin-1 (ANG-1)

Conditions tested - 14 days *in vitro*

(600,000 iTEC + 1,000 LMPPs + 100,000 WT MEFs) RTOC:
- No candidates added to RTOC media (4 technical replicates)
- 50,000 FTM cells added to RTOC / No candidates added to RTOC media (3 technical replicates)
- All candidates added to RTOC media (4 technical replicates)
- Group 1 candidates added to RTOC media (5 technical replicates)
- Group 2 candidates added to RTOC media (5 technical replicates)
- Group 3 candidates added to RTOC media (5 technical replicates)

(300,000 E15.5 TEC + 1,000 LMPPs + 100,000 WT MEFs) RTOC:
- No candidates added to RTOC media (3 technical replicates)

Figure 4.2.7. Selected candidate grouping and experimental conditions used to test whether Fetal Thymic Mesenchyme (FTM) function can be replaced in RTOC with candidates *in vitro*. Candidates were selected based on ligand-receptor analysis between FTM-TEC and WT MEFs-TEC. Candidates were selected based on ligand-receptor pairs with a higher correlation score in FTM-TEC than WT MEFs-TEC (Wilcoxon test, FDR<0.1). Candidates were further selected based on which were commercially available at Peprotech. All candidates were supplemented in the media at a concentration of 5 ng/ml in 2% FCS serum DMEM/F12+ 1% Penicillin/Streptomycin +1% GlutaMax . 50% of the media was supplemented every other day with the corresponding 2X candidate addition. RTOC were maintained *in vitro* and 14 days later were collected, digested and analysed by flow cytometry. Candidates were randomised to avoid bias and split into three groups based on limited tissue availability required to generate RTOC. This experiment was only carried out once and therefore data is preliminary.

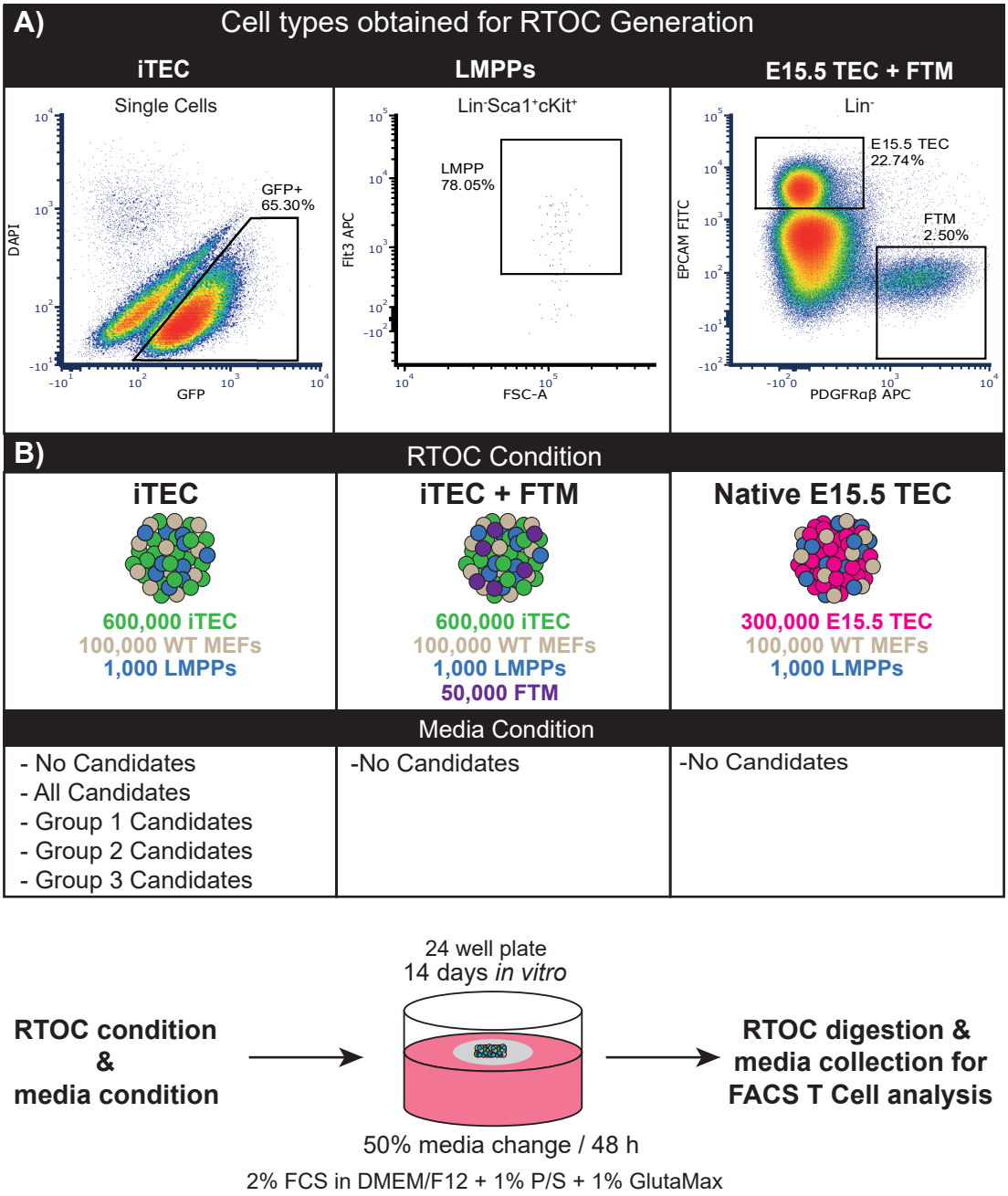


Figure 4.2.8. Experimental design for testing candidate ability to replace FTM in RTOC *in vitro*. A) Cell types obtained to generate RTOC to test candidates to replace FTM. iTEC (Day 18 GFP$^+$ iFoxn1 MEFs), Lymphoid primed multipotent progenitors (LMPPs: Lin$^-$ Sca1$^+$cKit$^+$Flt3$^+$), E15.5 TEC (Lin$^-$EpCAM$^+$), Fetal Thymic Mesenchyme (FTM: Lin$^-$PDGFRαβ$^+$). **B)** Experimental conditions used. All candidates were used at a 5 ng/ml concentration.

4.2.E. Group 2 candidates augment the function of iTEC-RTOC

Flow cytometry was used to determine whether supplementation of the culture medium with candidate factors affected thymopoiesis in iTEC-RTOC. The FACS panel used included a lineage cocktail (CD11b, CD11c, GR1, NK1.1, B220, TER119) to remove non-T lineage cells. CD45, CD24, TCRβ, CD4, CD8, 7-aminoactinomycin D (7-AAD), CD5, CD4 and CD8 were included to allow evaluation of T cell differentiation from DN to DP and then SP. CD5, a marker of TCR activation, was included to determine whether iTEC-RTOC support normal T cell differentiation from DP to SP, which requires TCR activation and positive selection to occur. Representative FACS plots of technical replicates for each condition and gating strategy implemented are shown in Figures 4.2.9-17. Samples were run on an ACEA NovoCyte® and the absolute cell counts of T cells (Lin^-CD45^+), DN ($CD4^-CD8^-$), DP ($CD4^+CD8^+$), SP4 ($CD4^+CD8^-$), SP8 ($CD4^-CD8^+$), Q1 ($CD24^-TCR\beta^-$), Q2($CD24^+TCR\beta^-$), Q3($CD24^+TCR\beta^+$), Q4($CD24^-TCR\beta^+$), $CD5^+$ DN, $CD5^+$ DP, $CD5^+$ SP4 and $CD5^+$ SP8 populations were collected for each technical replicate (Table 4.2.3). The mean absolute cell counts for each condition were calculated and plotted for each cell population, to detect trends in the conditions (Figure 4.2.18). Native E15.5 TEC-based RTOC data were not included in these graphs as the cell numbers generated were higher than the iTEC RTOC conditions, but cell counts are shown in Table 4.2.3.

The data were considered in terms of three processes: DN to SP progression; ability of T cells to progress from immature (Q1) through to mature (Q4) stages; and TCR activation based on expression of CD5 from DN through to SP. All of the data discussed were generated from a single experiment, and therefore must be repeated for confirmation of the conclusions drawn.

The data presented in Figures 4.2.11-18 show clear differences in outcome for the different groups. Medium supplementation with Group 2 factors resulted in the highest numbers and proportion of DP after 14 days of culture. Indeed, this condition outperformed FTM. Next progression to the SP4 stage was almost identical between Group 2 and +FTM with all other conditions generating notably lower cell numbers. Transition to the SP8 stage was most successful in Group 2, again outperforming +FTM.

CD24 expression occurs during early T cell development. High CD24 expression occurs during the DN stage followed by a decrease when progressing though to DP,

indicating undergoing selection into Immature SP, and complete downregulation when progressing to SP4 or SP8 T cells (Hough *et al*., 1994). CD24 upregulation is therefore an indication that β-selection has occurred, while CD24 downregulation indicates maturation of T cells. Again, iTEC-RTOC cultured with Group 2 factors presented the highest levels of $CD24^{+}TCR\beta^{-}$ cells, followed by iTEC-RTOC+FTM. Group 2 also presented the highest number of $CD24^{+}TCR\beta^{+}$ cells (averaging 2,650 cells), outperforming all other groups and suggesting support through β-selection. Lastly very few cells (<8) were present in the $CD24^{-}TCR\beta^{+}$ mature T cell stage in all conditions tested, including iTEC+FTM-RTOC, and indeed the cell numbers in this quadrant in the E15.5 TEC-RTOC were low compared to those in the four-week-old thymus.

CD5 expression is a direct indicator of pre-TCR and TCR signalling activation occurring during thymopoiesis and can therefore be used to indicate thymocytes that have undergone β- and positive selection. The lowest levels are found on DN T cells, followed by an increase at the DP and both SP4 and SP8 stages of T cell development (Fowlkes *et al*., 1985; Azzam *et al*., 1998). Again, supplementation of the iTEC-RTOC medium with Group 2 factors resulted in the highest proportion of cells $CD5^{+}$ DP, $CD5^{+}$ SP4 and $CD5^{+}$ SP8 cells, outperforming even iTEC+FTM-RTOC.

Overall supplementation of the iTEC-RTOC medium with Group 2 factors performed the best of all the test conditions in terms of the increased generation of DPs, SPs, Q3 Immature SPs and positive selection, outperforming iTEC+FTM-RTOC. These promising preliminary results suggest that the effect on iTEC-RTOC elicited by FTM cells can be replaced by the addition of factors in the media.

Group 2 contained the following eight candidates: Platelet factor 4 (PF4 or CXCL4), pleiotrophin (PTN), vascular endothelial growth factor A (VEGF-A), bone morphogenetic protein-4 (BMP-4), transforming growth factor β-2, angiopoietin like protein-7 (ANGPTL-7), interleukin-16 (IL-16) and insulin-like growth factor-2 (IGF-II). Many of these factors have been hypothesised or shown to play a role in the thymus. PF4 is a CXC chemokine that binds to heparin and has been linked to immune response regulation (Liu *et al*., 2005). PTN is another heparin binding protein known to induce epithelial, endothelial and MEF cell proliferation, associated with cancer (Fang *et al*., 1992). PTN has also been previously shown to be expressed by $Sca1^{+}$ thymic mesenchymal cells in the embryonic thymus and is

hypothesised to act on the PTPRZ1 receptor expressed on UEA1^{-} TEC (Dumont-Lagacé *et al*., 2017).

The role of VEGF-A in angiogenesis is well established and has been demonstrated to be required for normal thymus vascularisation (Müller *et al*., 2005). More recently a research group identified that VEGF acts directly on thymic stromal compartments eliciting pleiotropic effects through its receptors VEGFR2 and NRP1, with inhibition after birth resulting in transcriptional thymic maturation and a drop in thymocyte proliferation (de Barros *et al*., 2020). Our ligand-receptor analysis suggests that VEGF-A may interact with NRP1 expressed on TEC.

BMP signaling is crucial for thymic morphogenesis, acts on stroma not lymphocytes in the thymus and is also known to regulate *Foxn1* induction and is expressed by early fetal TEC (Bleul and Boehm, 2005). Specifically, production of BMP-4 by endothelial cells acts on TEC to increase *Foxn1* expression inducing thymic regeneration (Wertheimer *et al*., 2018).

Inhibition of TGF-β slows thymic involution and this effect is due to direct action on TEC through TGFβRII, rather than on lymphocytes (Hauri-Hohl *et al*., 2008). TGF-β2 expressed at high levels inhibits LSK (lin^{-}Sca1^{++}kit^{+}) haematopoietic progenitor cell proliferation, however, it displayed a stimulatory role at low concentrations (Langer *et al*., 2004).

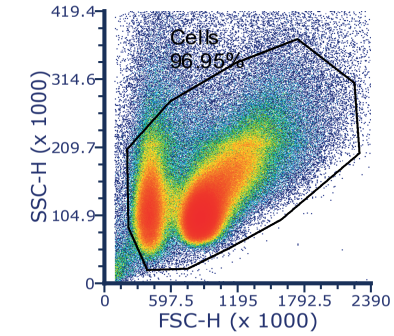
Angiopoietin-like proteins are used in haematopoietic stem and progenitor cell (HSPCs) cultures to induce proliferation by acting on Notch (Lin *et al*., 2015).

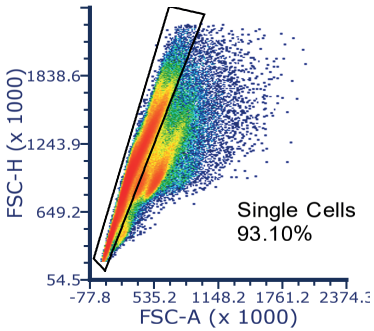
Interleukin-16 (formerly known as lymphocyte chemoattractant factor) was the first chemoattractant to be identified to act and recruit CD4^{+} T cells and has a role in T cell proliferation (Center and Cruikshank, 1982; Tuzova *et al*., 2015).

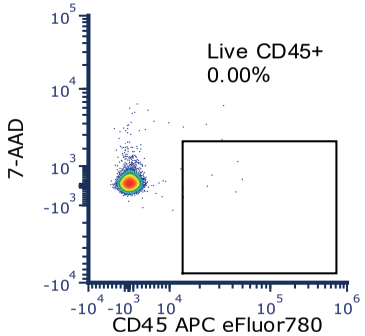
Transgenic mouse studies have shown that IGF-II acts on TEC through inducing proliferation and differentiation and impacts laminin and fibronectin components of the ECM which mediate interactions between TEC and developing T cells (Savino *et al*., 2005; Mendes-da-Cruz *et al*., 2018). Previous studies examining IGF-II found that T cell proliferation and differentiation in FTOC were also inhibited by anti-IGFII and anti-IGF-IR monoclonal antibodies, or an anti-M6P/IGF-IIR polyclonal antibody (Kecha *et al.*, 2000).

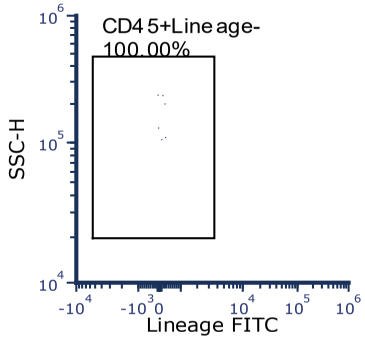
These results need to be replicated to confirm the conclusion that Group 2 factors can replace FTM in iTEC-RTOC. In addition, the Group 2 factors must be tested alone or in smaller groups, and at different concentrations.

It is also important to note that testing of the candidate factors in groups may mask the effect of some factors, if other factors in the group have an inhibitory effect on RTOC function. Non-the-less, these findings are a first step in understanding how to replace FTM.

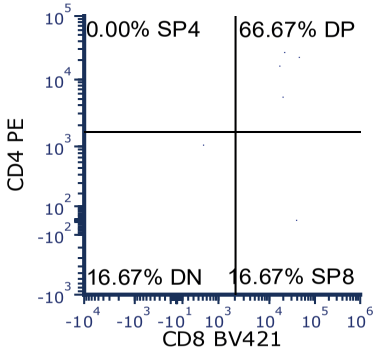








Gated on CD45+Lineage-



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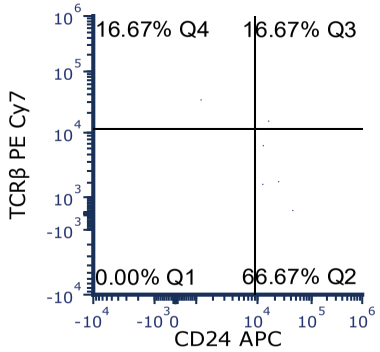

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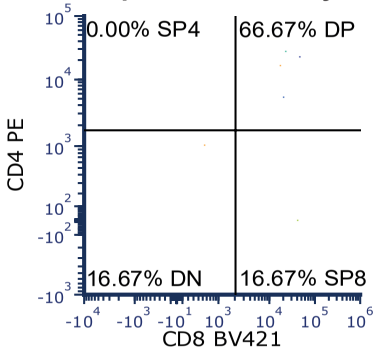

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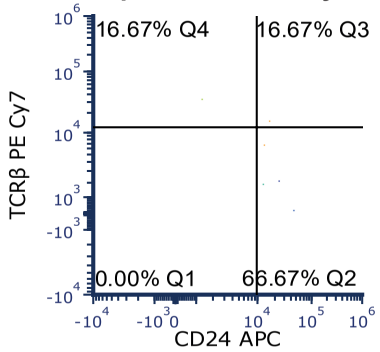
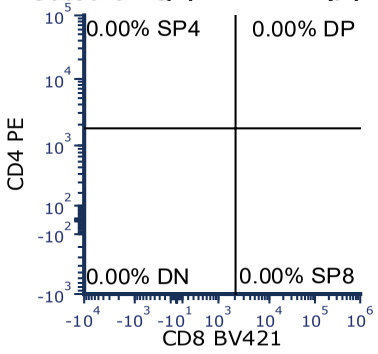
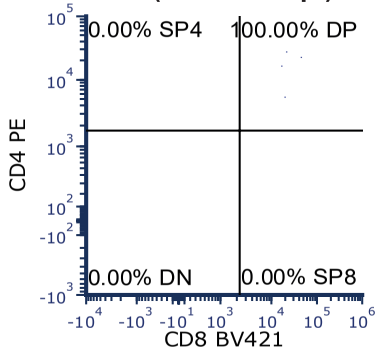


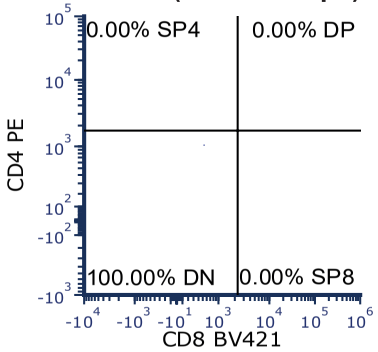
Gated on Q1(CD24-TCRβ-)



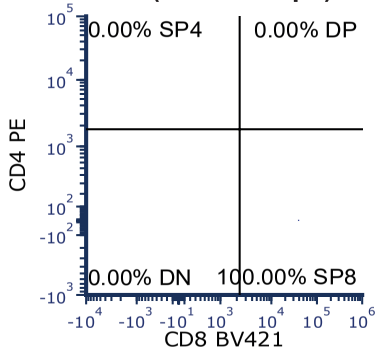
Gated on Q2(CD24+TCRβ-)



Gated on Q3(CD24+TCRβ+)



Gated on Q4(CD24-TCRβ+)



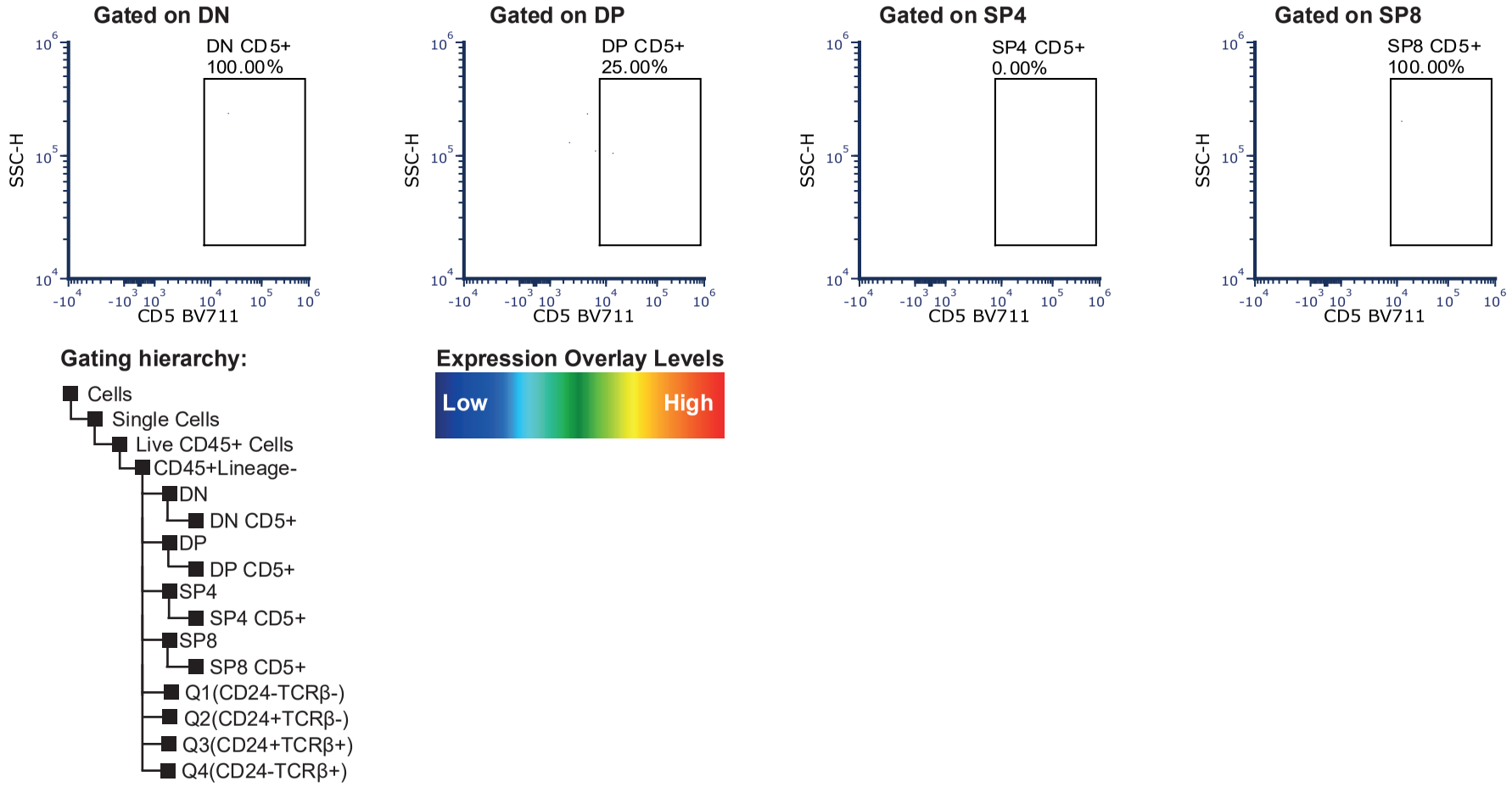
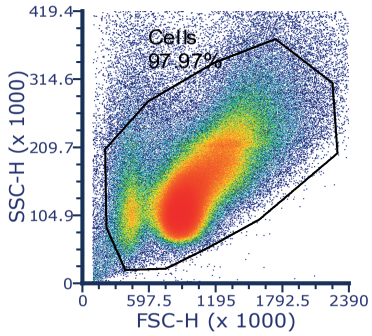
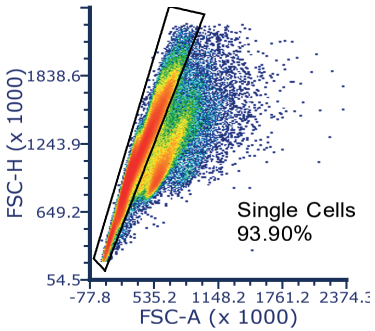
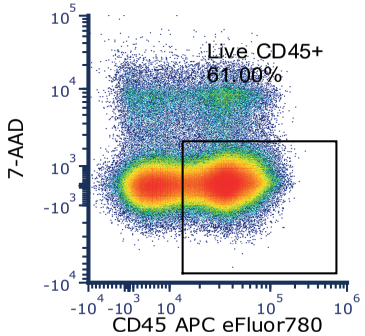
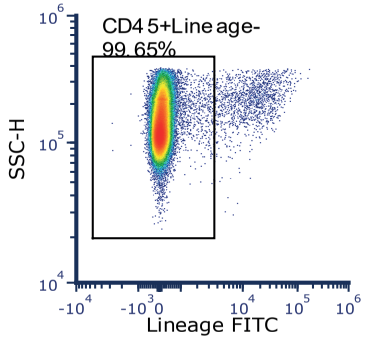


Figure 4.2.9. Validation of the staining panel used for assessing thymopoiesis in iTEC-RTOC: unstained WT thymus. Representative FACS plots and gating hierarchy. Lineage FITC cocktail (CD11b, CD11c, GR1, NK1.1, B220, TER119). Unstained sample underwent the same enzymatic digestion as the RTOC samples analysed for this experiment. N=1 experiment.

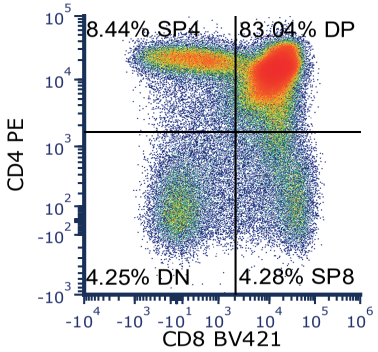








Gated on CD45+Lineage-



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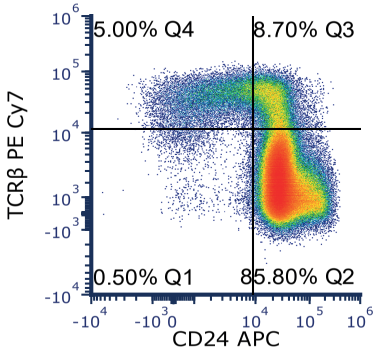
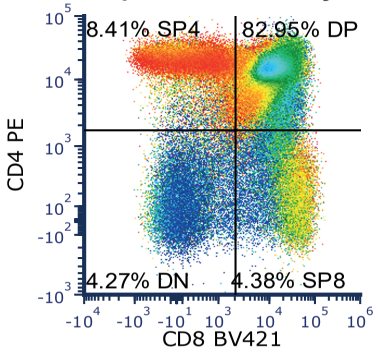
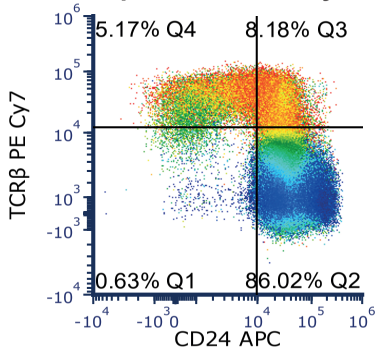


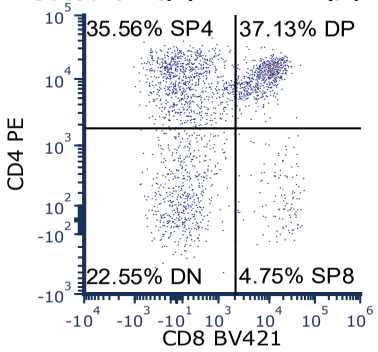
CD5 Expression Overlay



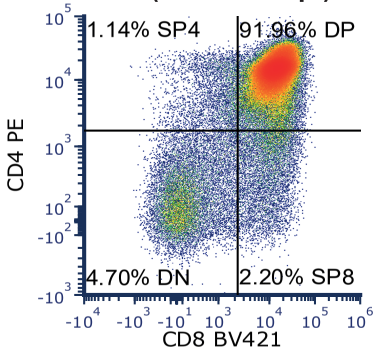
CD5 Expression Overlay



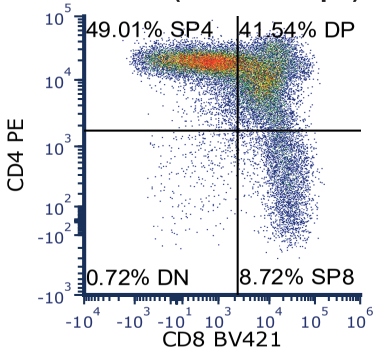
Gated on Q1(CD24-TCRβ-)



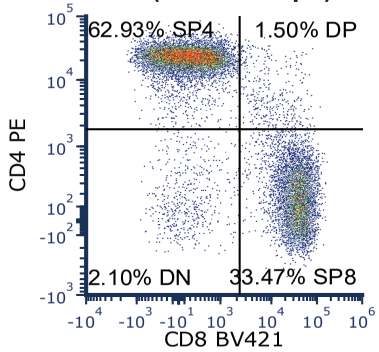
Gated on Q2(CD24+TCRβ-)



Gated on Q3(CD24+TCRβ+)



Gated on Q4(CD24-TCRβ+)



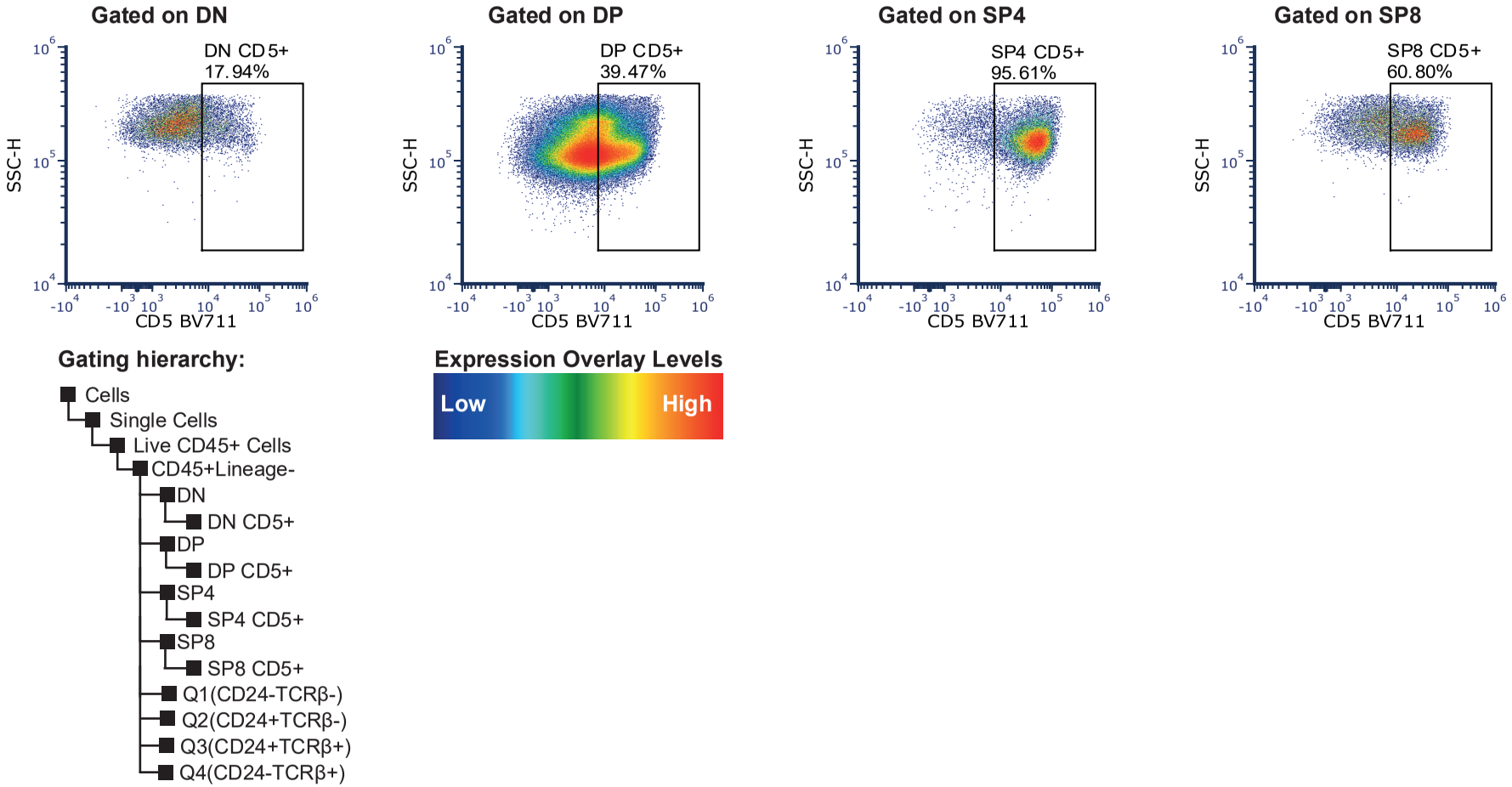
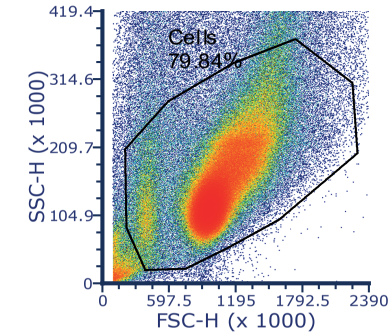
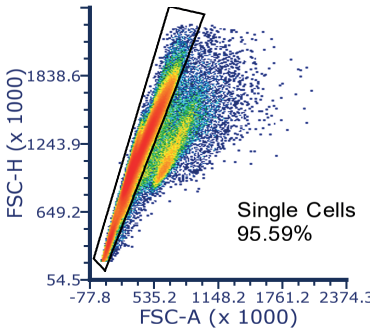
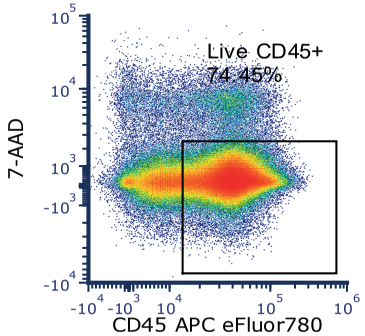
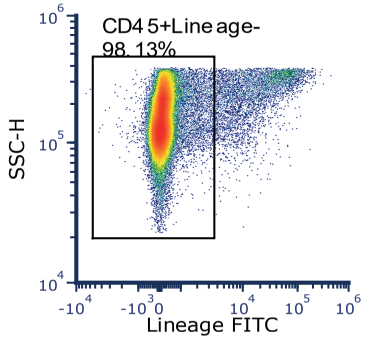


Figure 4.2.10. Validation of the staining panel used for assessing thymopoiesis in iTEC-RTOC: 4 week old WT thymus. Representative FACS plots and gating hierarchy. Lineage FITC cocktail (CD11b, CD11c, GR1, NK1.1, B220, TER119). WT thymus T Cells underwent the same enzymatic digestion as the RTOC samples analysed for this experiment. N=1 experiment.

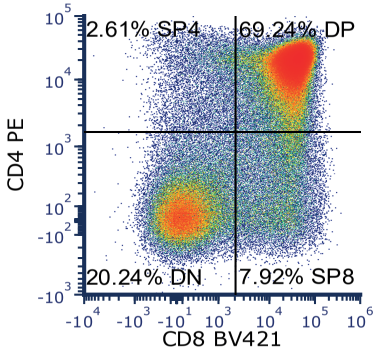








Gated on CD45+Lineage-



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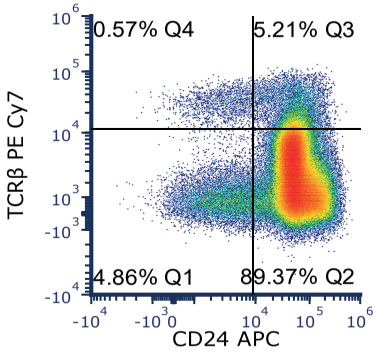
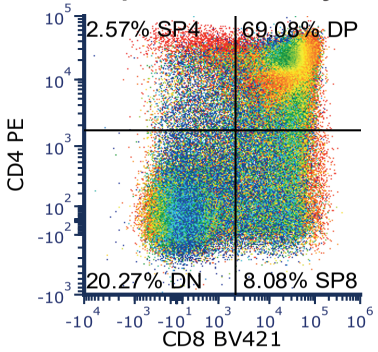


CD5 Expression Overlay



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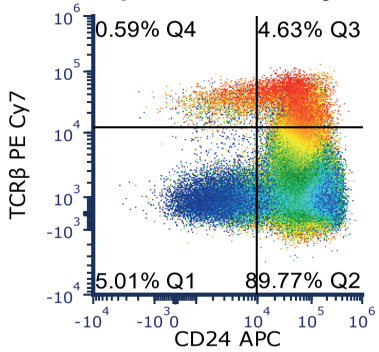
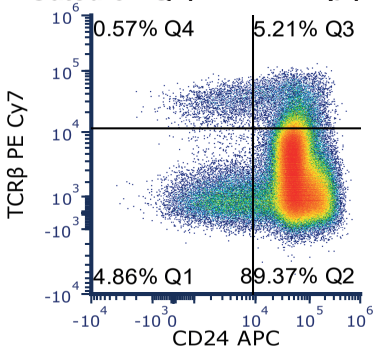
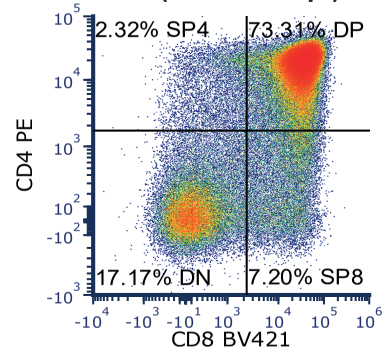


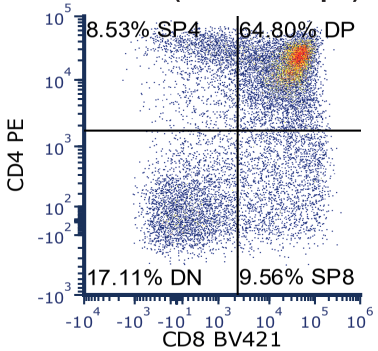
Gated on Q1(CD24-TCRβ-)



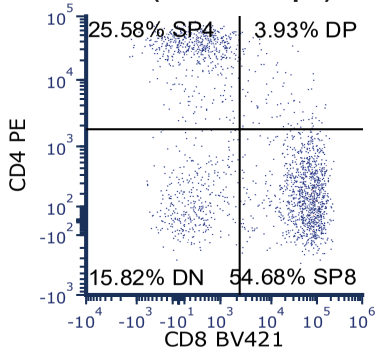
Gated on Q2(CD24+TCRβ-)



Gated on Q3(CD24+TCRβ+)



Gated on Q4(CD24-TCRβ+)



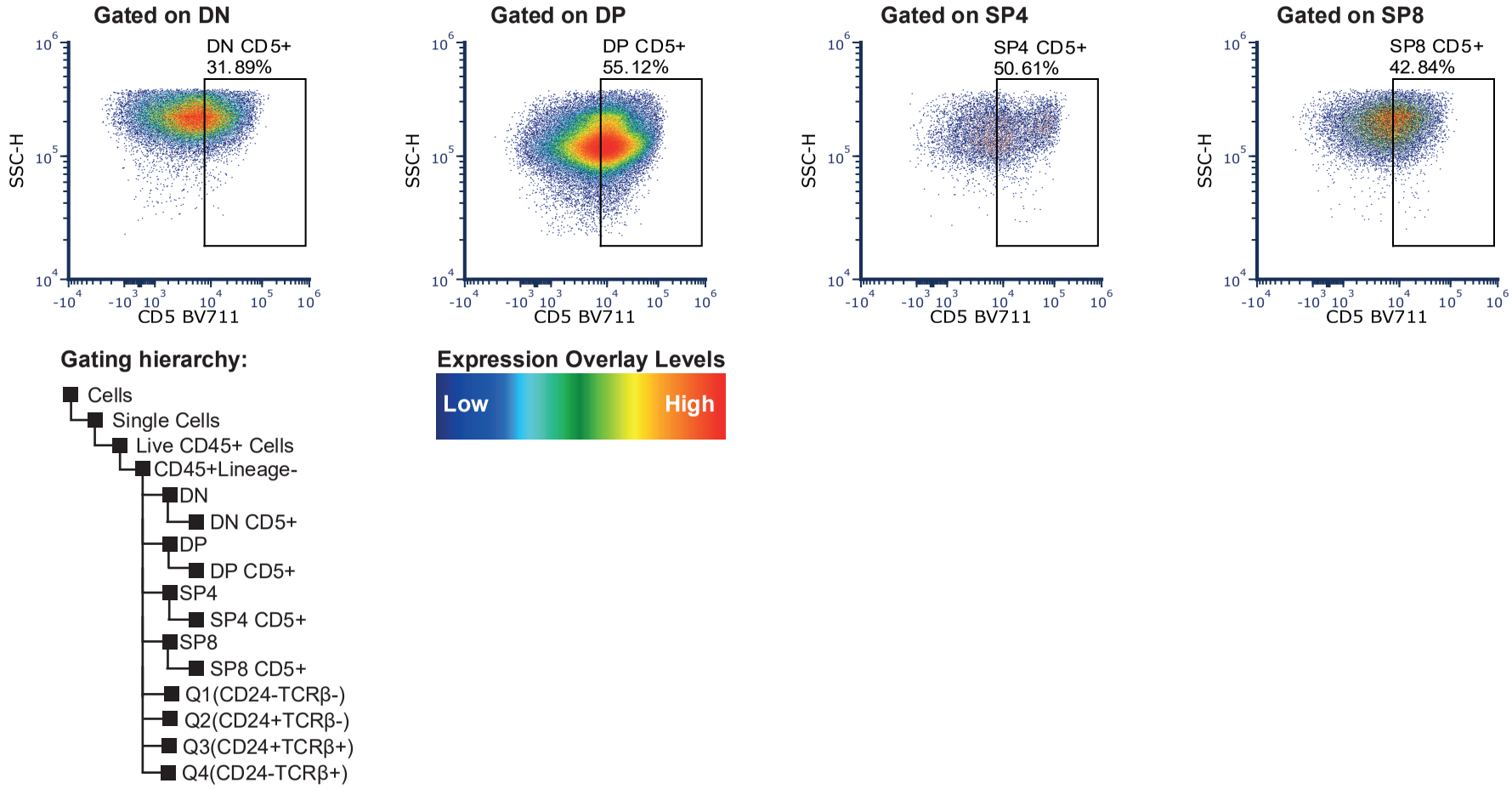
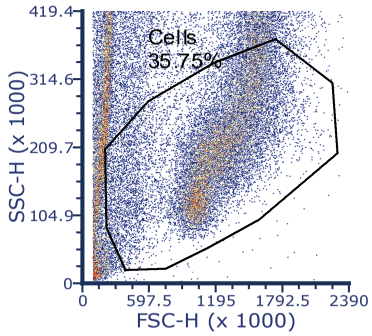
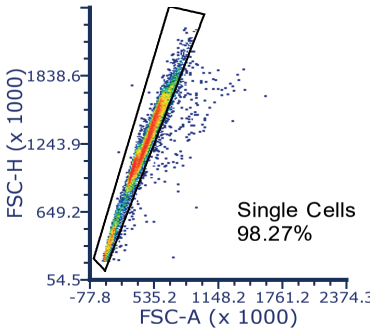
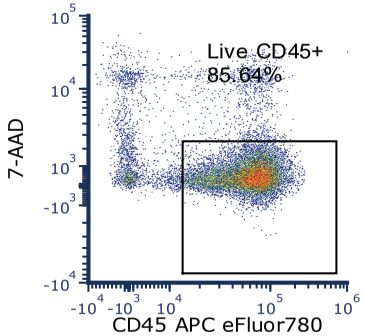
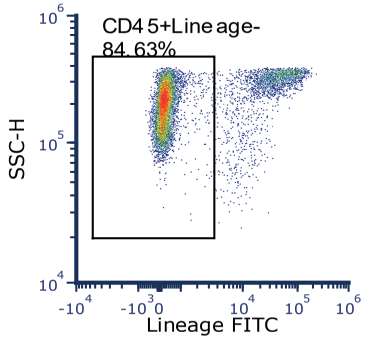


Figure 4.2.11. Validation of staining panel used for assessing thymopoiesis in iTEC-RTOC: E15.5 TEC RTOC 14 days *in vitro*. Representative FACS plots of technical replicates and gating hierarchy. Lineage FITC cocktail (CD11b, CD11c, GR1, NK1.1, B220, TER119). Native E15.5 TEC-based RTOC (300,000 Native E15.5 TEC + 1,000 LMPPs + 100,000 WT MEFs) cultured in media containing no candidates added for 14 days. N=1 experiment.

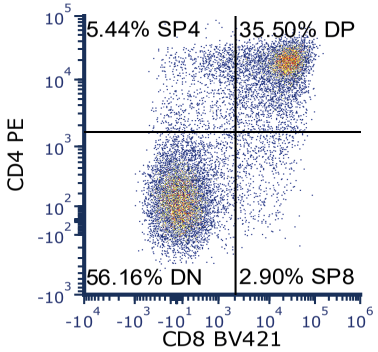








Gated on CD45+Lineage-



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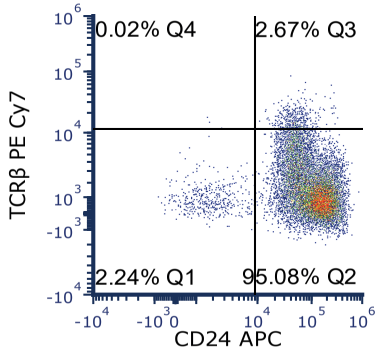
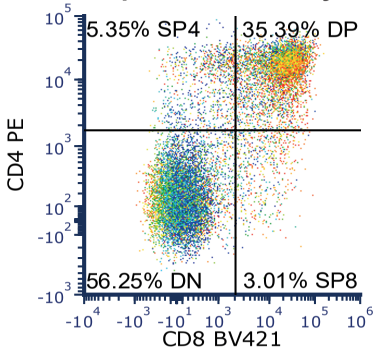
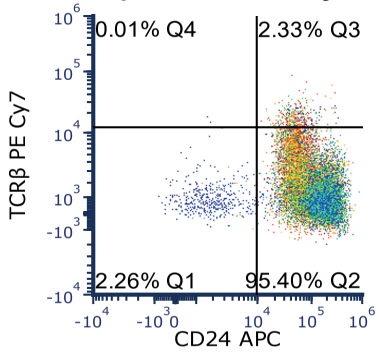


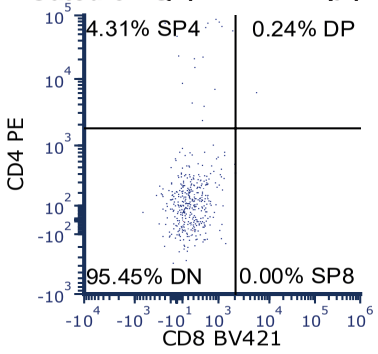
CD5 Expression Overlay



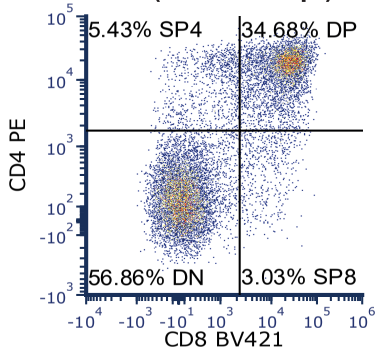
CD5 Expression Overlay



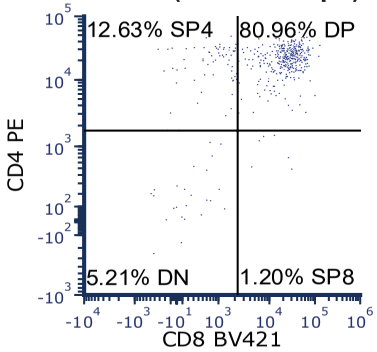
Gated on Q1(CD24-TCRβ-)



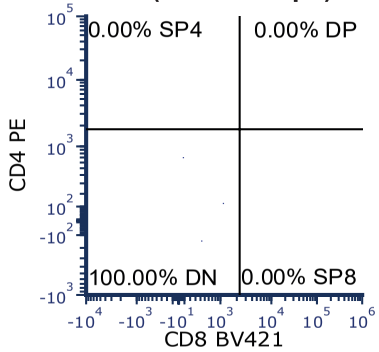
Gated on Q2(CD24+TCRβ-)



Gated on Q3(CD24+TCRβ+)



Gated on Q4(CD24-TCRβ+)



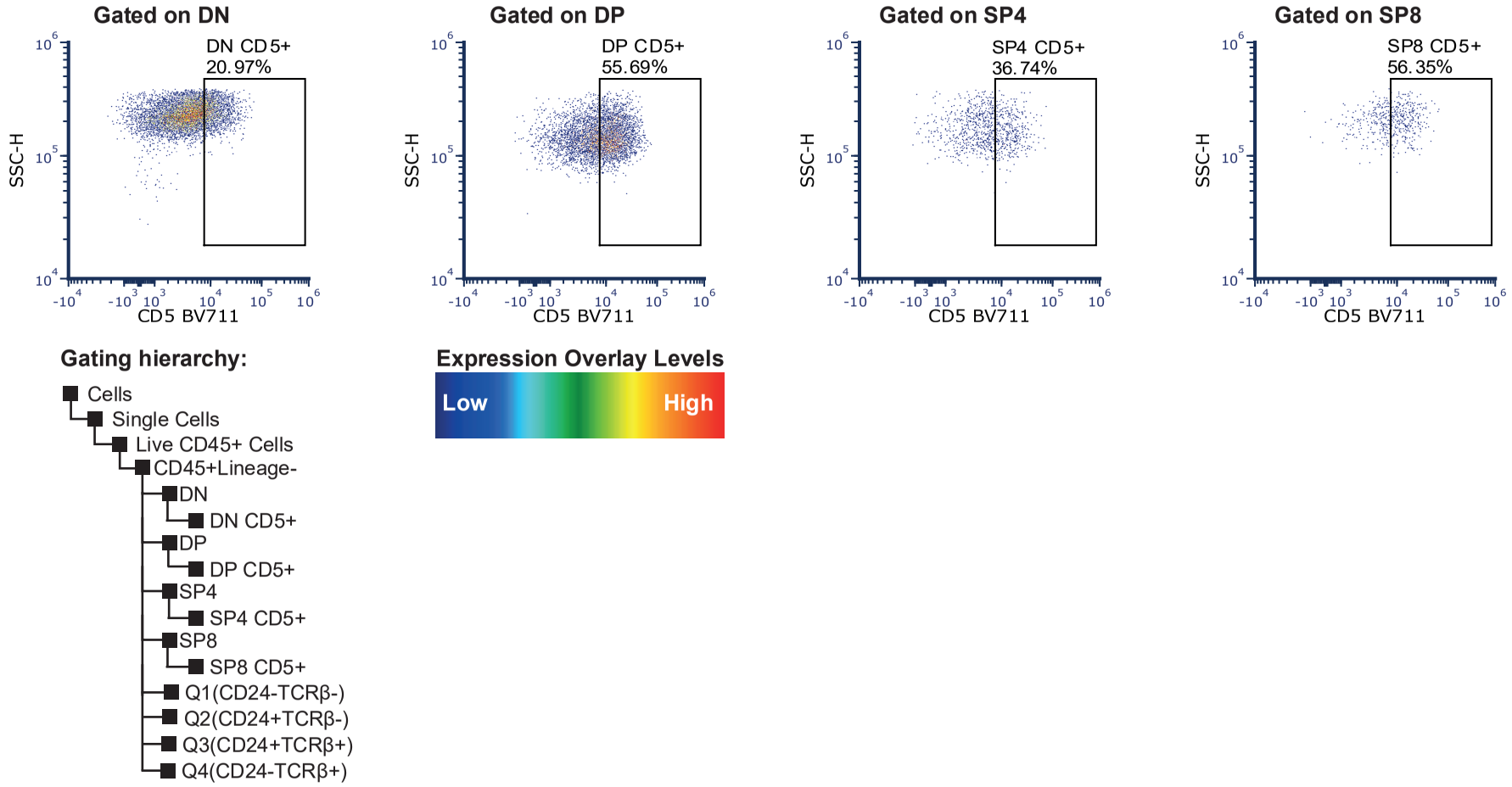
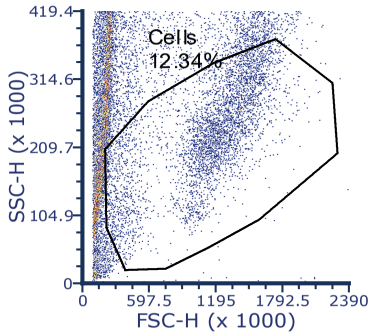
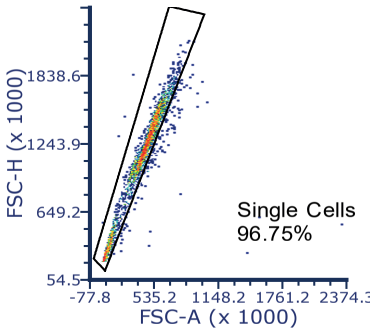
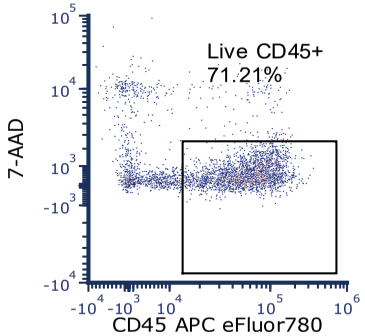
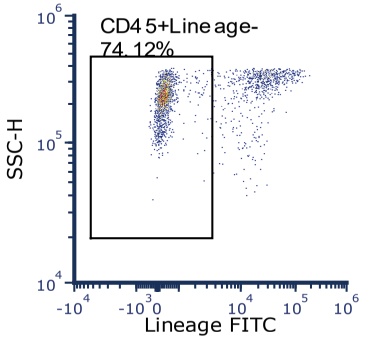


Figure 4.2.12. iTEC+FTM-RTOC 14 days *in vitro*. Representative FACS plots of technical replicates and gating hierarchy. Lineage FITC cocktail (CD11b, CD11c, GR1, NK1.1, B220, TER119). iTEC+FTM RTOC (600,000 iTEC + 1,000 LMPPs + 100,000 WT MEFs + 50,000 FTM) cultured in media containing no candidates added for 14 days. N=1 experiment.

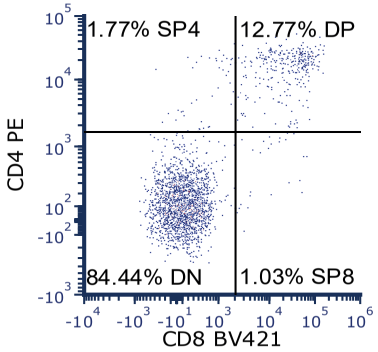








Gated on CD45+Lineage-



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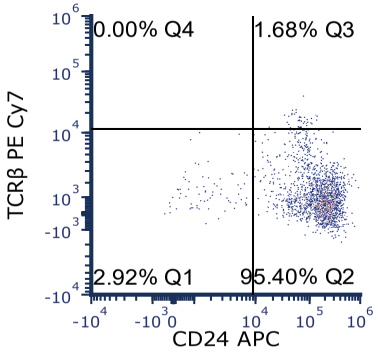

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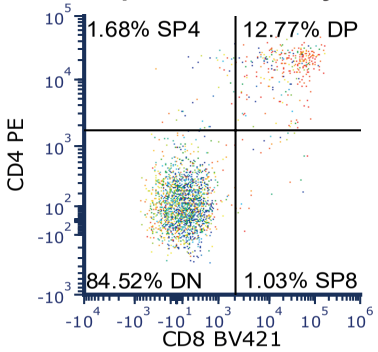

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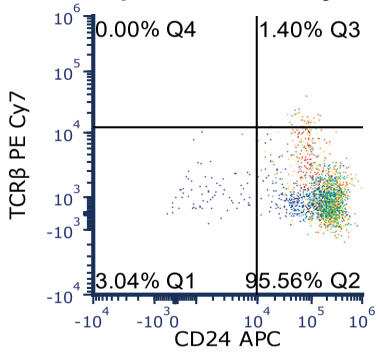

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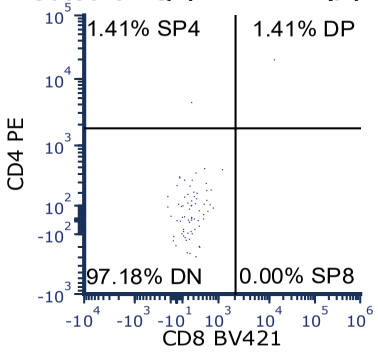
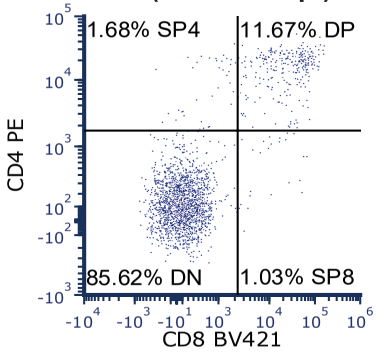
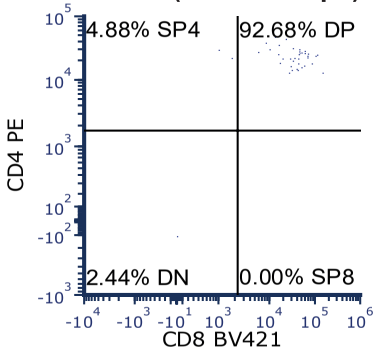


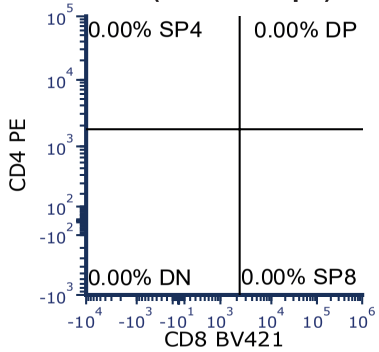
Gated on Q2(CD24+TCRβ-)



Gated on Q3(CD24+TCRβ+)



Gated on Q4(CD24-TCRβ+)



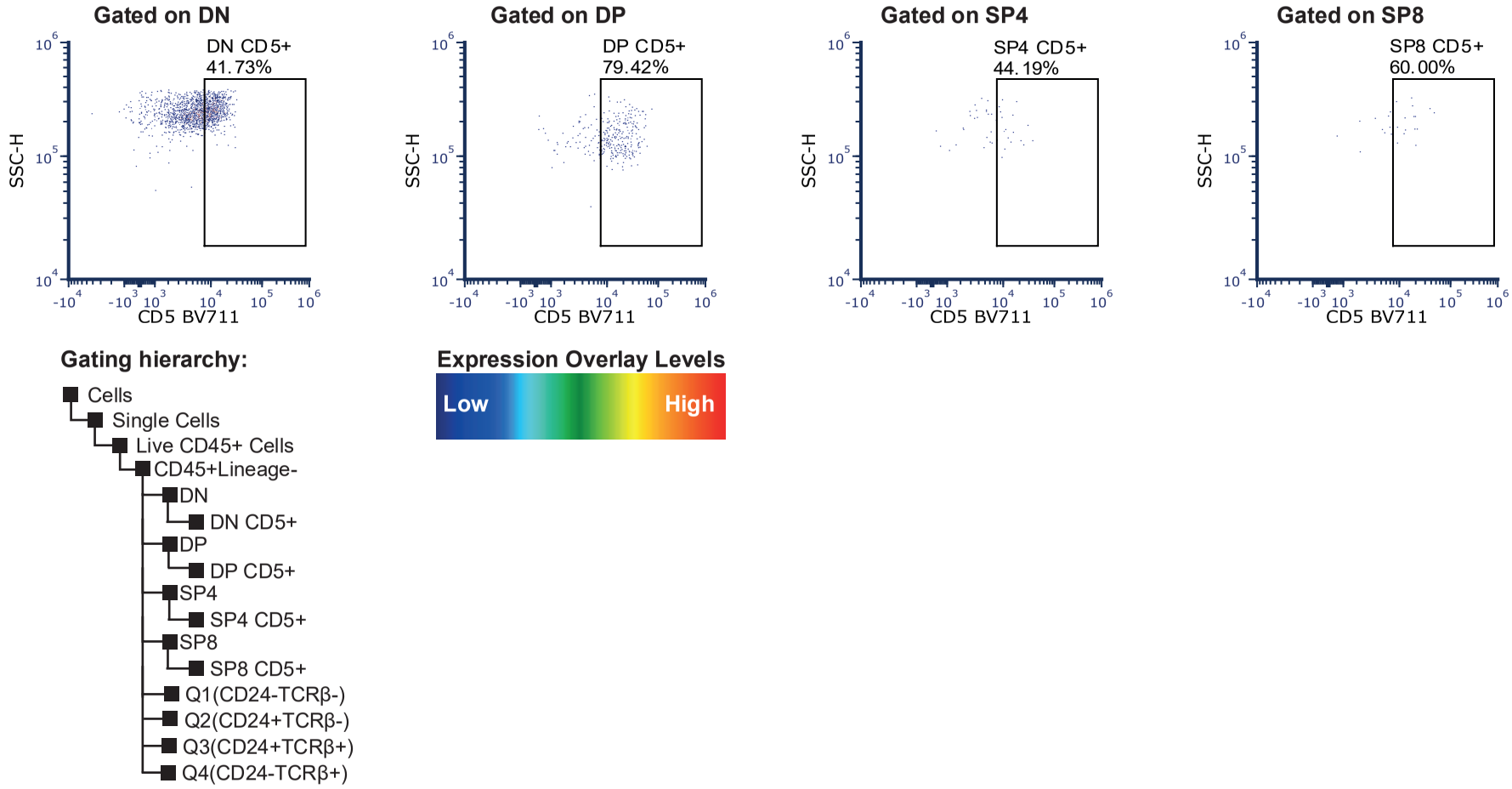
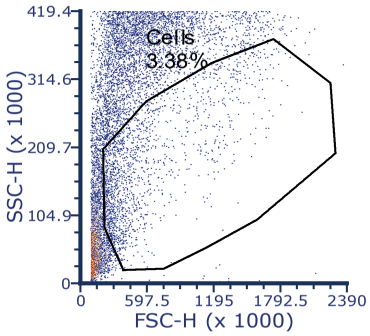
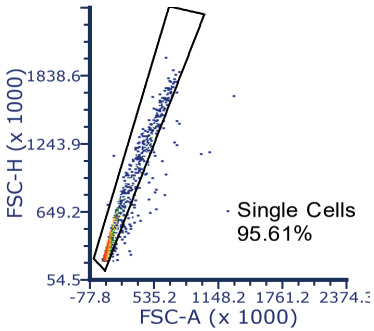
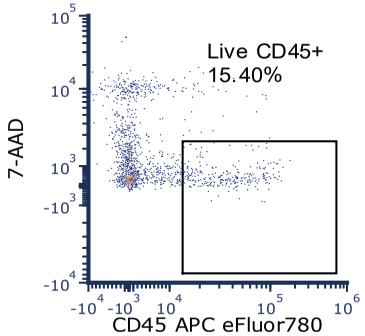
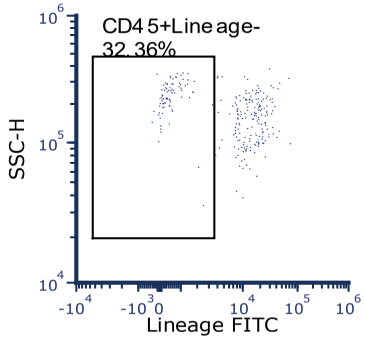


Figure 4.2.13. iTEC-RTOC with no candidates supplemented 14 days *in vitro.* Representative FACS plots of technical replicates and gating hierarchy. Lineage FITC cocktail (CD11b, CD11c, GR1, NK1.1, B220, TER119). iTEC RTOC (600,000 iTEC + 1,000 LMPPs + 100,000 WT MEFs) cultured in media not supplemented with selected candidates. N=1 experiment.









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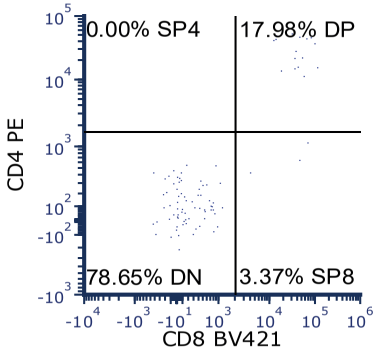

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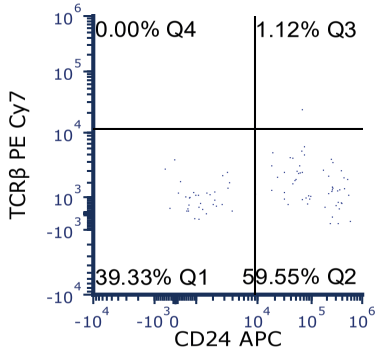
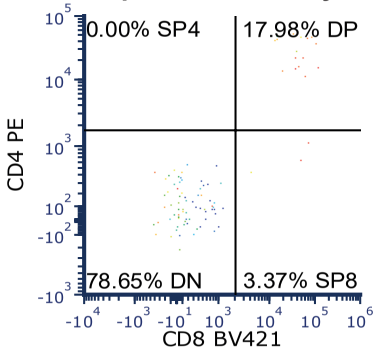


CD5 Expression Overlay



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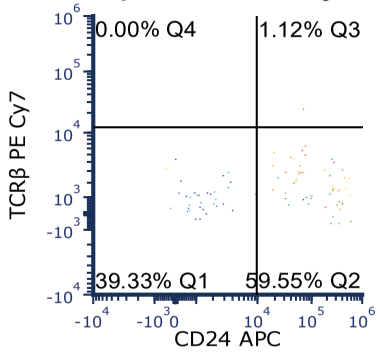
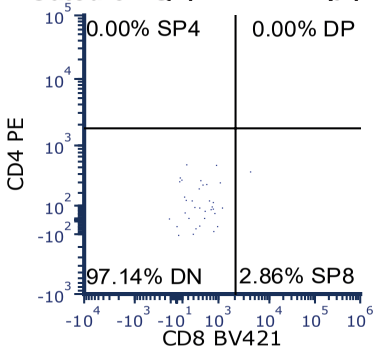


Gated on Q1(CD24-TCRβ-)



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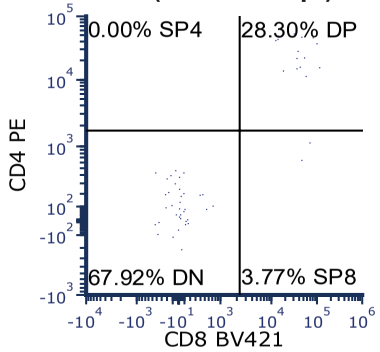
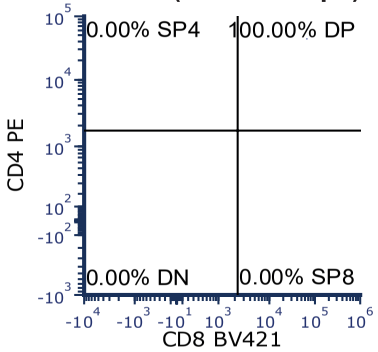
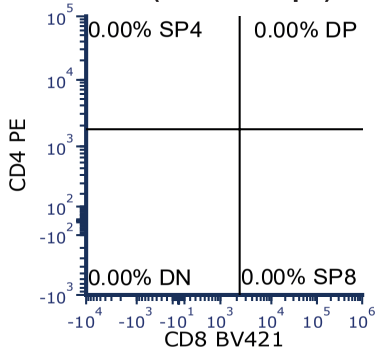


Gated on Q3(CD24+TCRβ+)



Gated on Q4(CD24-TCRβ+)



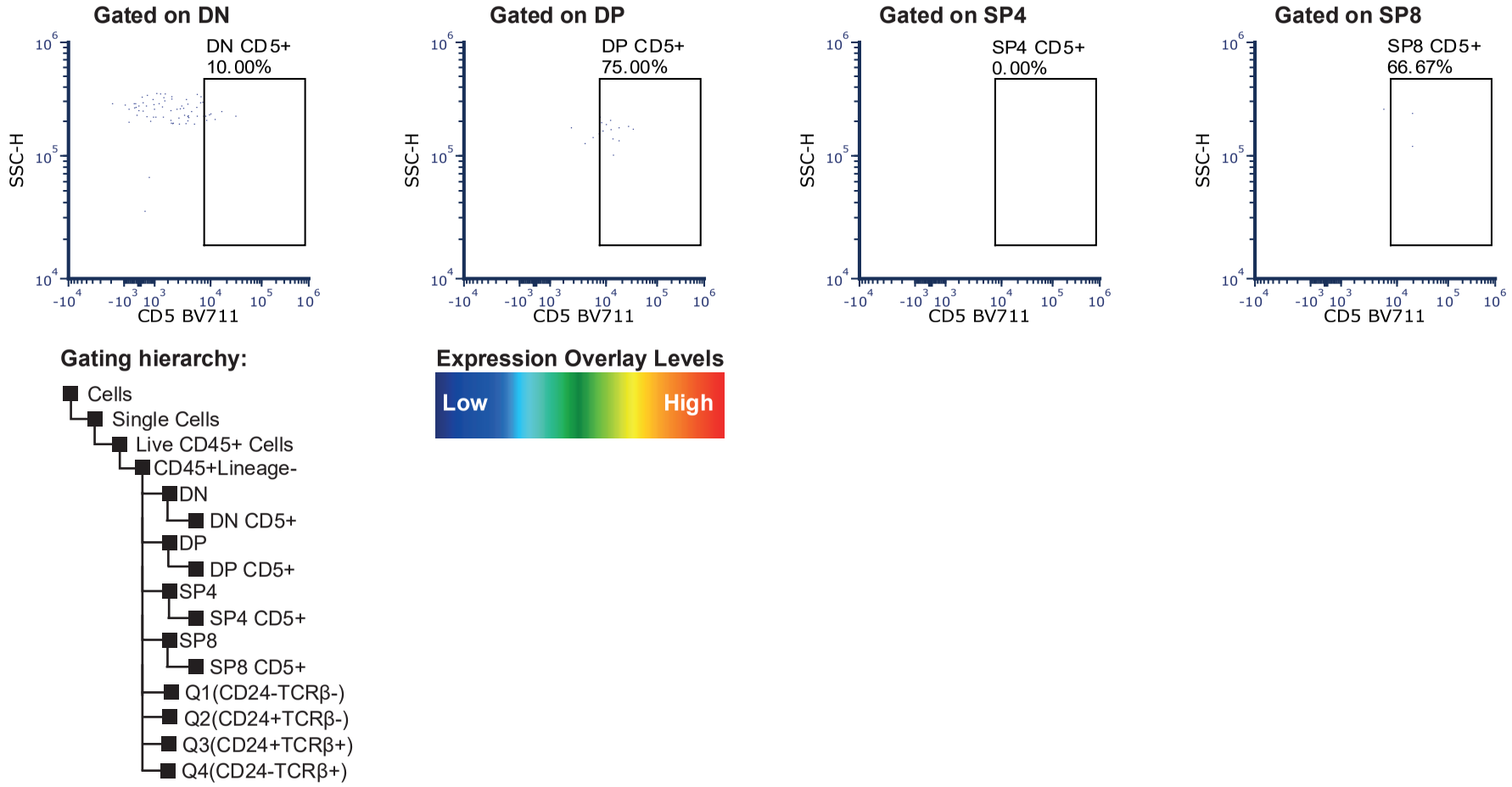
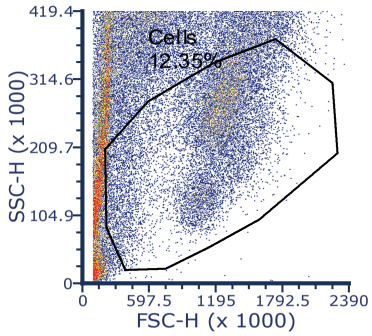
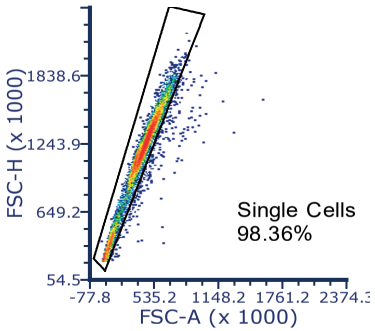
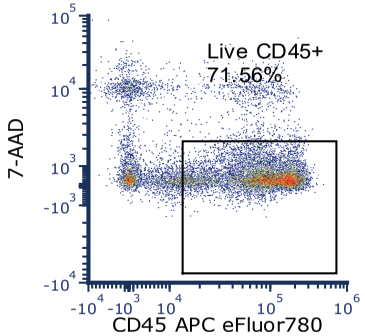
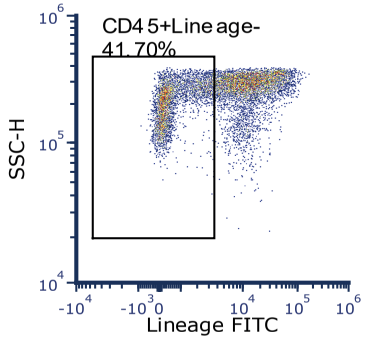


Figure 4.2.14. iTEC-RTOC supplemented with all selected candidates 14 days *in vitro.* Representative FACS plots of technical replicates and gating hierarchy. Lineage FITC cocktail (CD11b, CD11c, GR1, NK1.1, B220, TER119). iTEC RTOC (600,000 iTEC + 1,000 LMPPs + 100,000 WT MEFs) cultured in media supplemented with all 25 candidates each at 5 ng/ml concentration for 14 days. N=1 experiment.

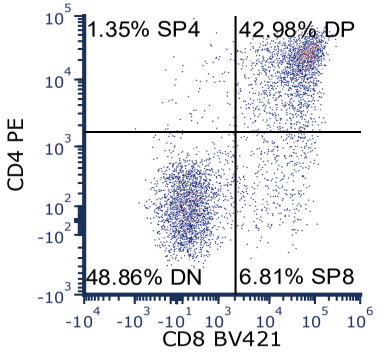




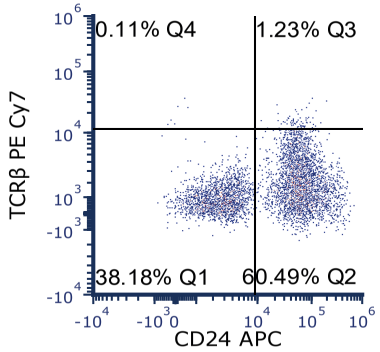




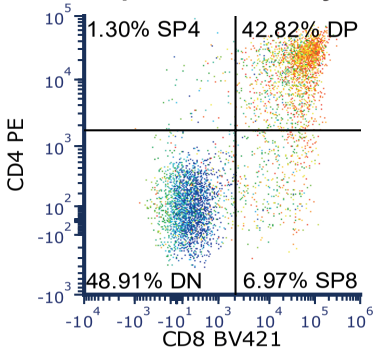
Gated on CD45+Lineage-



Gated on CD45+Lineage-



CD5 Expression Overlay



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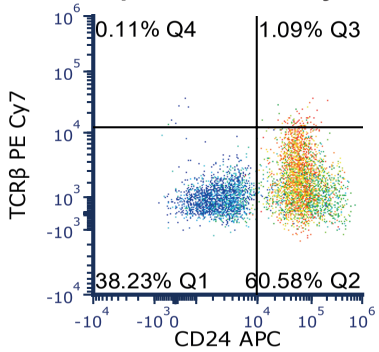
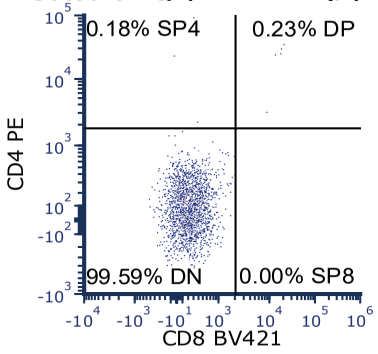
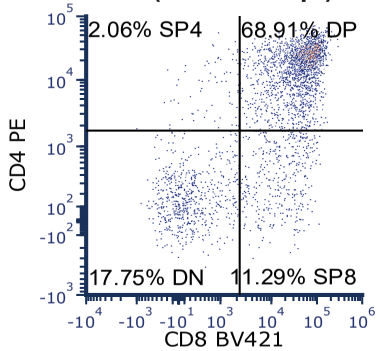


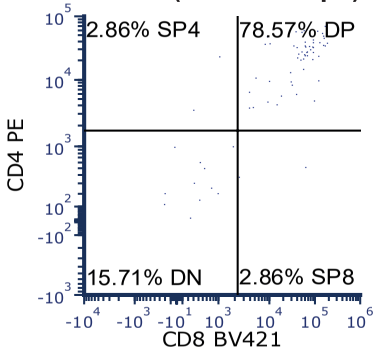
Gated on Q1(CD24-TCRβ-)



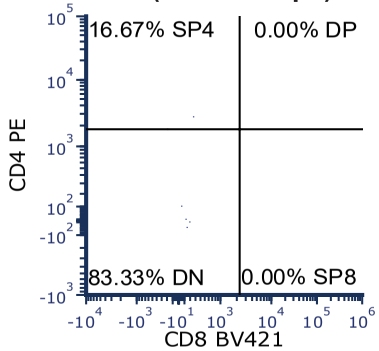
Gated on Q2(CD24+TCRβ-)



Gated on Q3(CD24+TCRβ+)



Gated on Q4(CD24-TCRβ+)



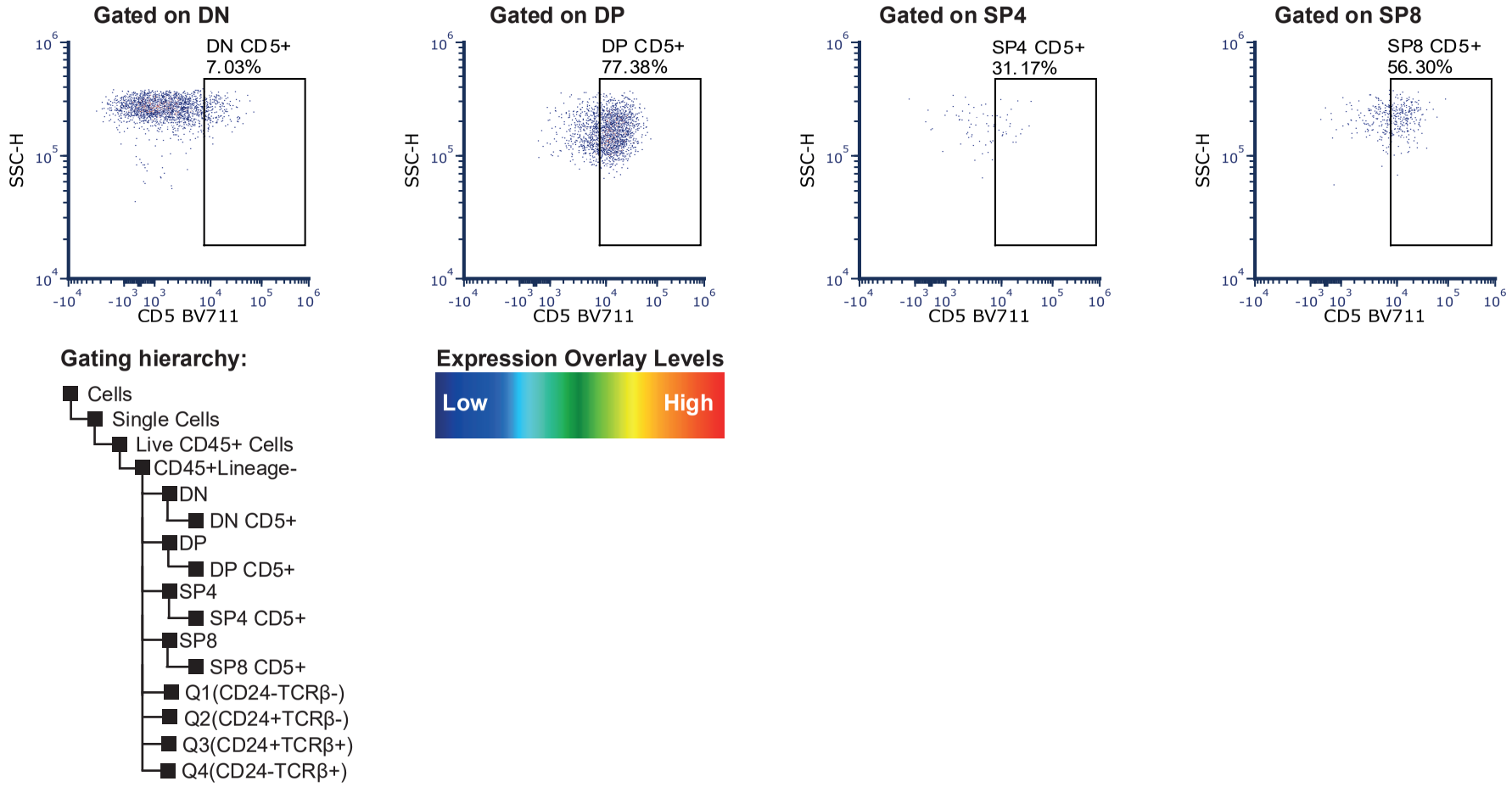
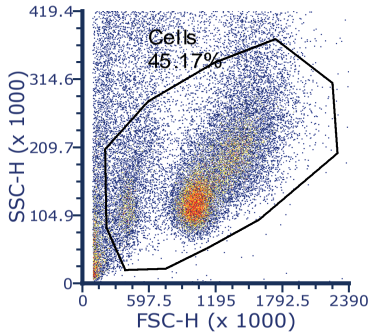
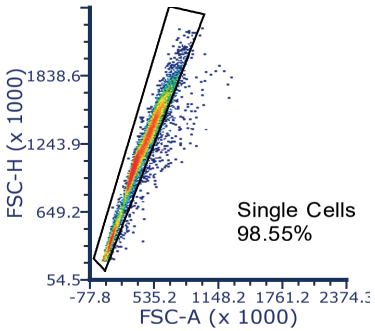
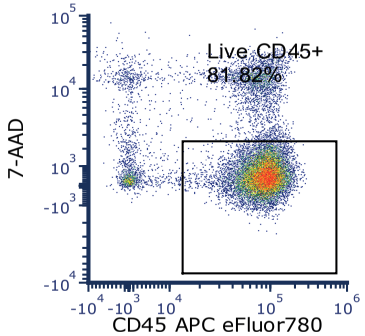
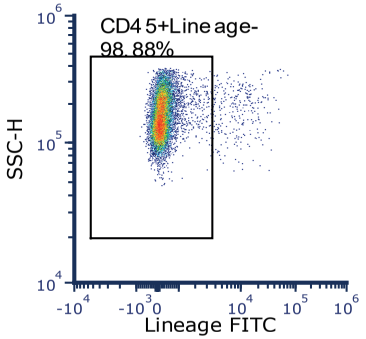


Figure 4.2.15. iTEC-RTOC supplemented with Group 1 candidates 14 days *in vitro.* Representative FACS plots of technical replicates and gating hierarchy. Lineage FITC cocktail (CD11b, CD11c, GR1, NK1.1, B220, TER119). iTEC RTOC (600,000 iTEC + 1,000 LMPPs + 100,000 WT MEFs) cultured in media supplemented with Group 1 candidates (HGF, IL-15, TSLP, IGF-1, FGF-1, BMP-2, PGF, EGF, MK) each at a concentration of 5 ng/ml. N=1 experiment.









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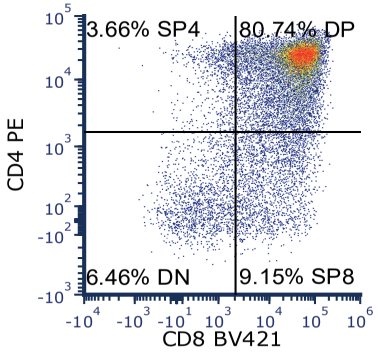

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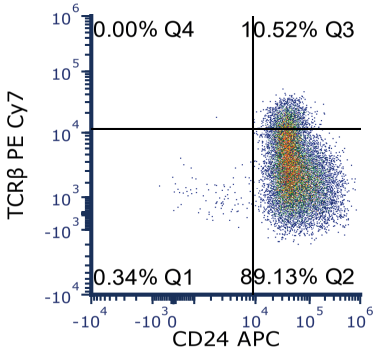
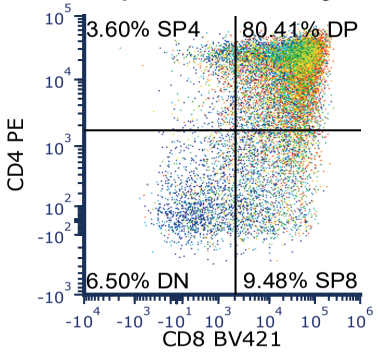
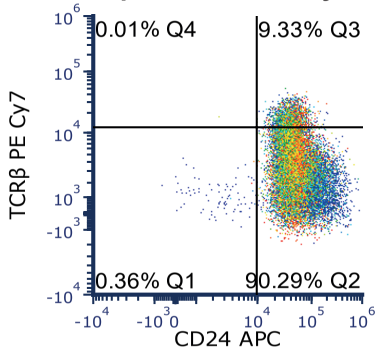


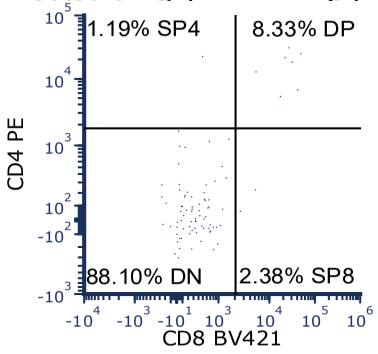
CD5 Expression Overlay



CD5 Expression Overlay



Gated on Q1(CD24-TCRβ-)



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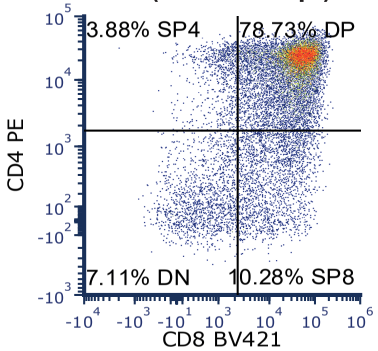

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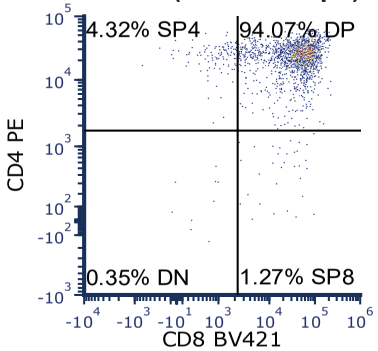
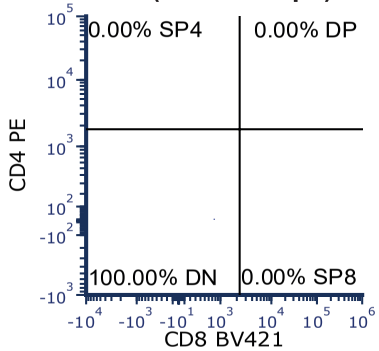


Gated on Q4(CD24-TCRβ+)



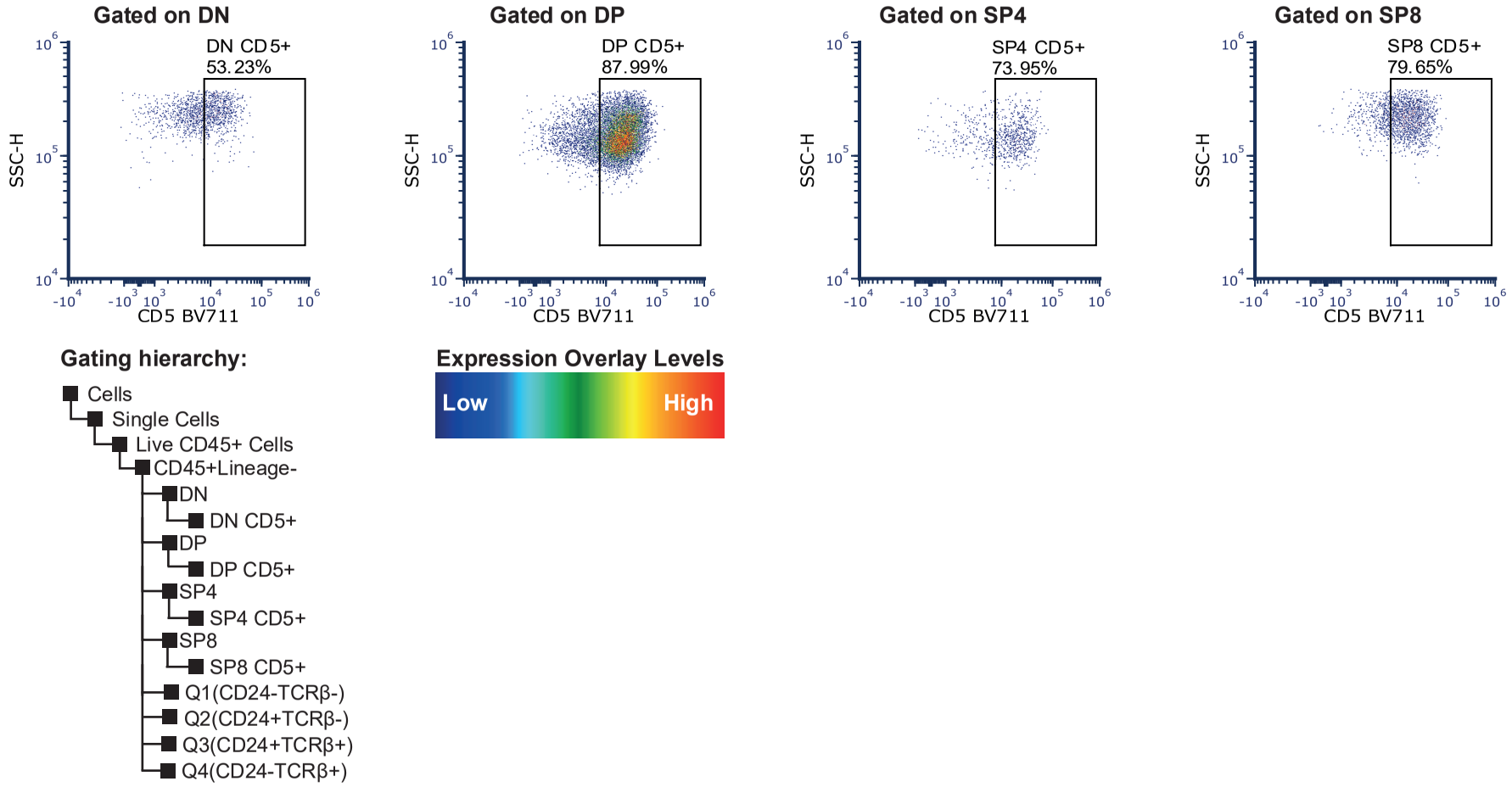
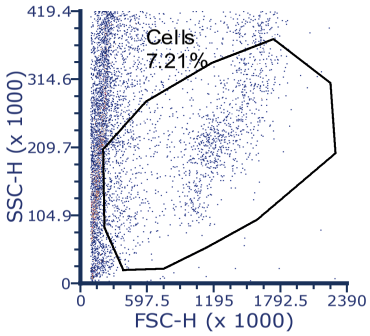
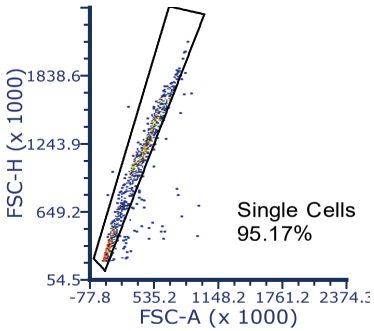
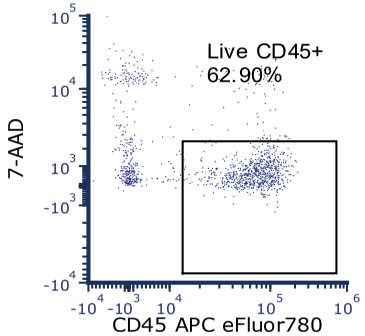
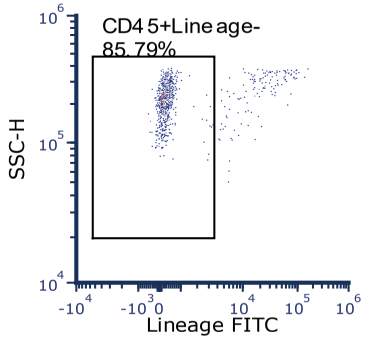


Figure 4.2.16. iTEC-RTOC supplemented with Group 2 candidates 14 days *in vitro*. Representative FACS plots of technical replicates and gating hierarchy. Lineage FITC cocktail (CD11b, CD11c, GR1, NK1.1, B220, TER119). iTEC RTOC (600,000 iTEC + 1,000 LMPPs + 100,000 WT MEFs) cultured in media supplemented with Group 2 candidates (CXCL4, PTN, VEGF-A, BMP-4, TGF-β2, ANGPTL-7, IL-16,IGF-2) each at a concentration of 5 ng/ml. N=1 experiment.

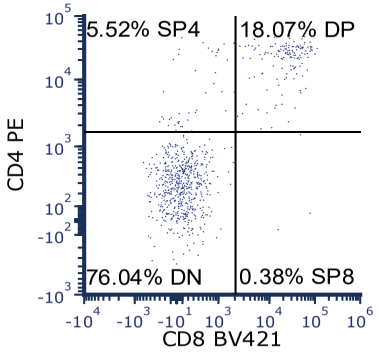




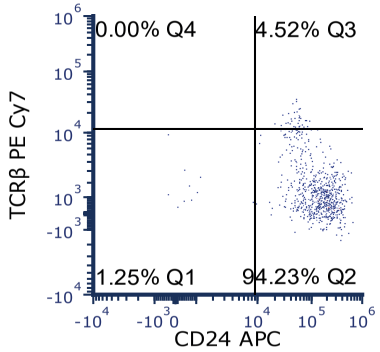




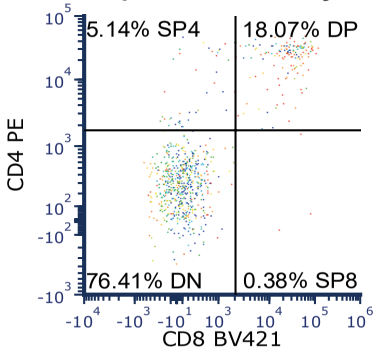
Gated on CD45+Lineage-



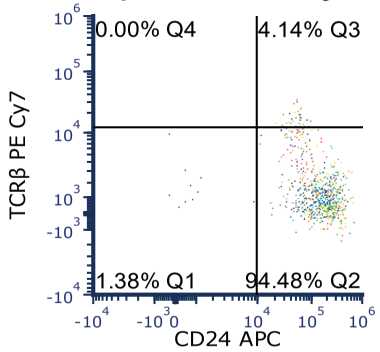
Gated on CD45+Lineage-



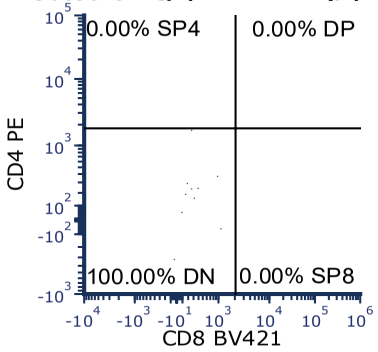
CD5 Expression Overlay



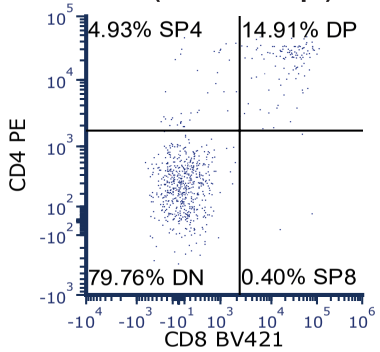
CD5 Expression Overlay



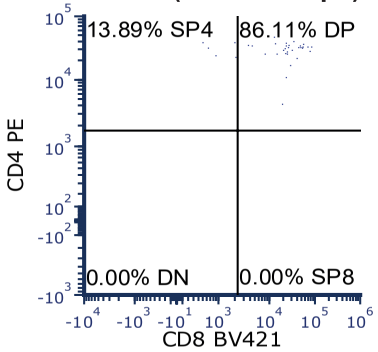
Gated on Q1(CD24-TCRβ-)



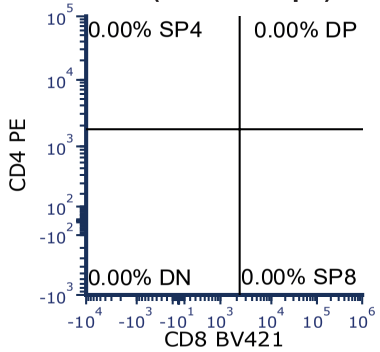
Gated on Q2(CD24+TCRβ-)



Gated on Q3(CD24+TCRβ+)



Gated on Q4(CD24-TCRβ+)



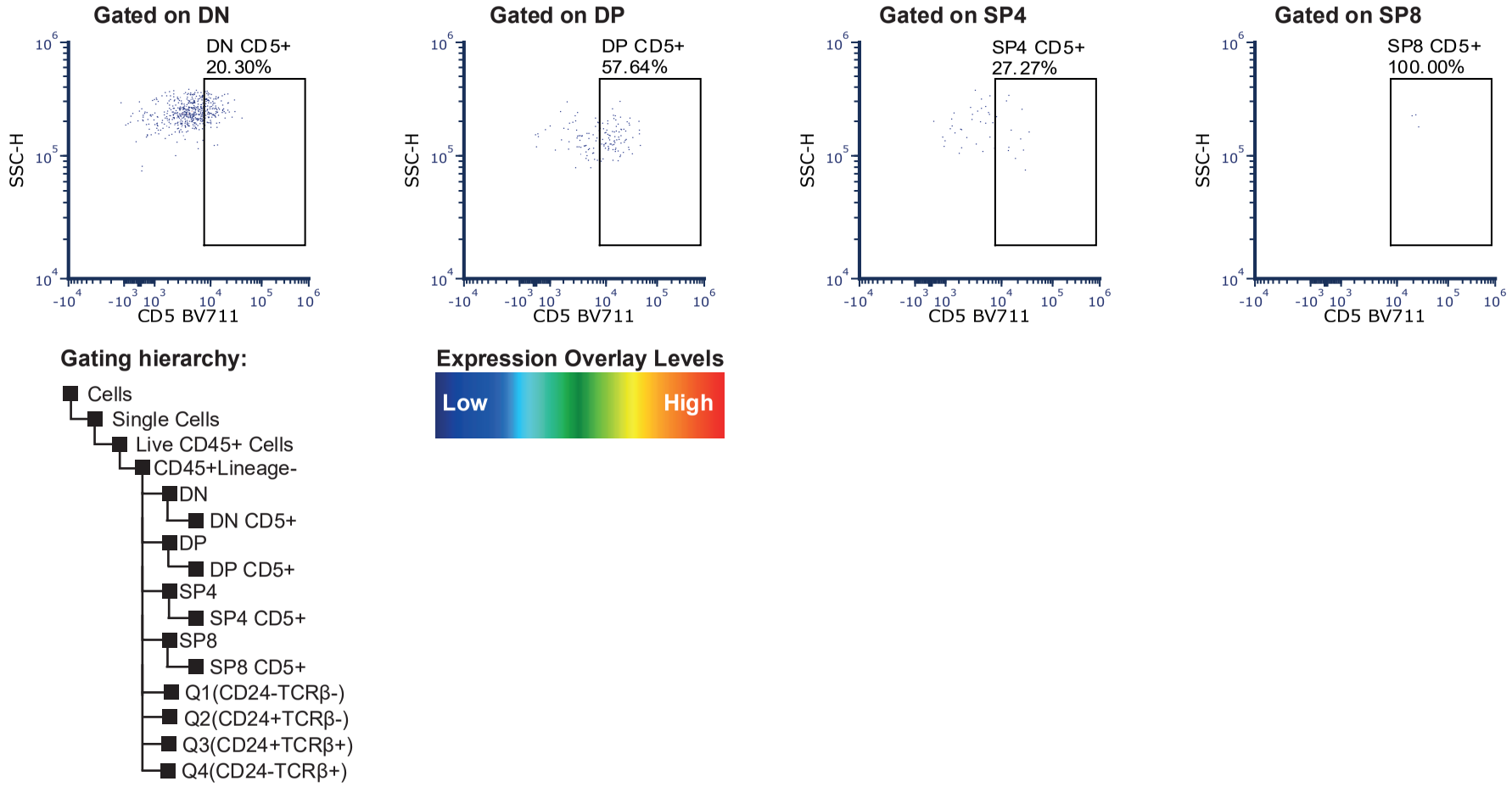
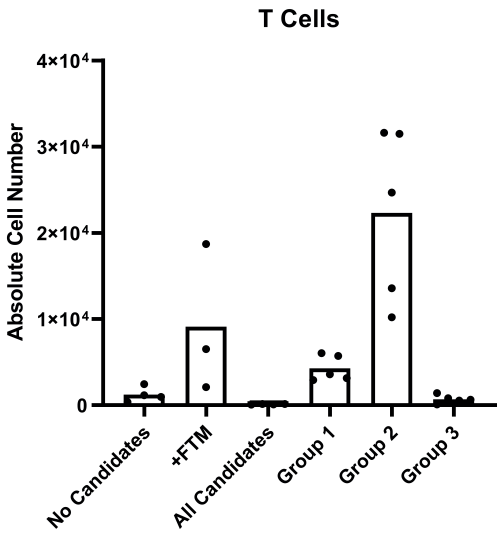


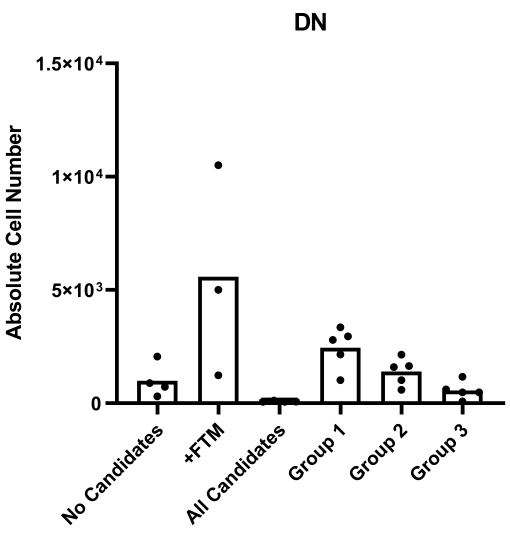
Figure 4.2.17. iTEC-RTOC supplemented with Group 3 candidates 14 days *in vitro*. Representative FACS plots of technical replicates and gating hierarchy. Lineage FITC cocktail (CD11b, CD11c, GR1, NK1.1, B220, TER119). iTEC RTOC (600,000 iTEC + 1,000 LMPPs + 100,000 WT MEFs) cultured in media supplemented with Group 3 candidates (IL-34, Slit2, FGF-18, IGFBP4, BMP-7, RARRES2, FGF-10, ANG-1) each at a concentration of 5 ng/ml. N=1 experiment.

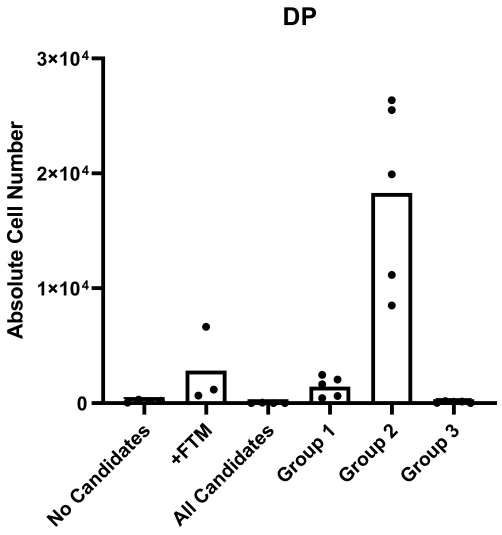
	Tech Rep	T Cells	DN	$CD5^+$ DN	DP	$CD5^+$ DP	SP4	$CD5^+$ SP4	SP8	$CD5^+$ SP8	Q1	Q2	Q3	Q4
No Candidates	1	1154	883	351	221	151	38	19	12	12	32	1083	38	1
	2	350	302	100	44	29	4	3	0	0	12	333	5	0
	3	947	718	264	203	121	16	7	10	7	31	877	39	0
	4	2435	2056	858	311	247	43	19	25	15	71	2323	41	0
	Mean	**1222**	**990**	**393**	**195**	**137**	**25**	**12**	**12**	**9**	**37**	**1154**	**31**	**0**
FTM	1	2106	1232	204	668	283	173	55	33	10	31	2015	59	1
	2	6507	5000	903	1179	656	218	71	110	61	130	6291	78	8
	3	18701	10502	2202	6638	3697	1018	374	543	306	418	17781	499	3
	Mean	**9105**	**5578**	**1103**	**2828**	**1545**	**470**	**167**	**229**	**126**	**193**	**8696**	**212**	**4**
All Candidates	1	125	64	0	47	35	2	0	12	5	64	59	1	1
	2	89	70	7	16	12	0	0	3	2	35	53	1	0
	3	129	120	0	5	3	2	0	2	0	114	15	0	0
	4	65	64	0	0	0	1	0	0	0	65	0	0	0
	Mean	**102**	**80**	**2**	**17**	**13**	**1**	**0**	**4**	**2**	**70**	**32**	**1**	**0**
Group 1	1	5710	2790	196	2454	1899	77	24	389	219	2180	3454	70	6
	2	6029	3354	313	2053	1753	77	43	545	384	2611	3231	183	4
	3	2916	1018	110	1637	1402	51	38	210	160	774	1952	189	1
	4	3587	2951	198	434	306	49	10	153	94	2426	1138	21	2
	5	3124	2148	260	627	518	44	17	305	191	1556	1527	40	1
	Mean	**4273**	**2452**	**215**	**1441**	**1176**	**60**	**26**	**320**	**210**	**1909**	**2260**	**101**	**3**
Group 2	1	31620	2139	1248	25512	22942	655	488	3314	2833	137	27599	3879	5
	2	10203	587	294	8502	7435	271	188	843	684	42	8957	1204	0
	3	31480	1638	1008	26369	24016	326	209	3147	2608	254	27207	4012	7

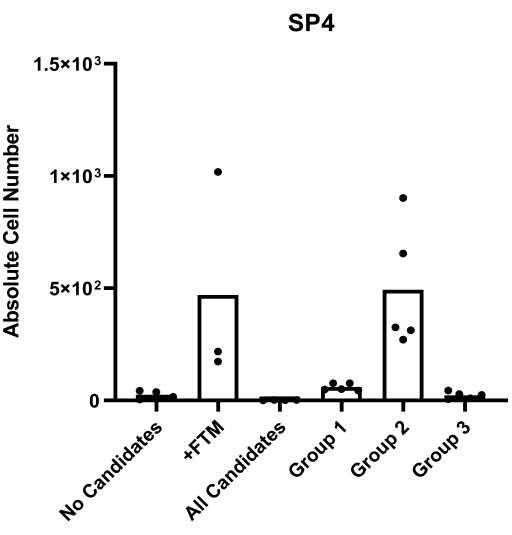
	4	24666	1593	848	19915	17524	902	667	2256	1797	84	21986	2595	1
	5	13578	1019	566	11157	9176	313	217	1089	833	35	11959	1581	3
	Mean	**22309**	**1395**	**793**	**18291**	**16219**	**493**	**354**	**2130**	**1751**	**110**	**19542**	**2654**	**3**
Group 3	1	1392	1165	207	178	79	28	9	21	9	61	1299	32	0
	2	90	80	4	4	1	6	0	0	0	27	63	0	0
	3	625	479	115	128	95	10	2	8	4	15	568	42	0
	4	539	475	118	30	14	25	2	9	7	114	420	3	2
	5	797	606	123	144	83	44	12	3	3	10	751	36	0
	Mean	**689**	**561**	**113**	**97**	**54**	**23**	**5**	**8**	**5**	**45**	**620**	**23**	**0**
TEC	1	391352	79195	25253	270956	149345	10207	5166	30994	13278	19004	349750	20385	2213
	2	14835	2031	488	11547	6119	448	211	809	355	570	13614	620	31
	3	385265	97760	27829	243429	139470	9809	5099	34267	12933	23354	343486	16479	1946
	Mean	**263817**	**59662**	**17857**	**175311**	**98311**	**6821**	**3492**	**22023**	**8855**	**14309**	**235617**	**12495**	**1397**

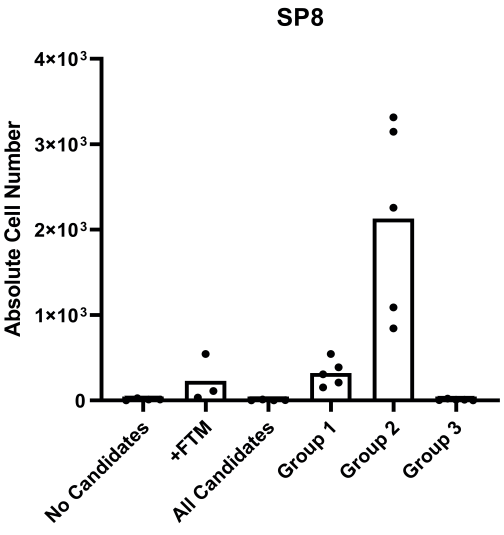
Table 4.2.3. Absolute cell counts of each cell population in all candidate screen conditions. Detailed account of absolute cell numbers obtained from each technical replicate along with mean per condition. Native TEC RTOC utilised E15.5 WT TEC.

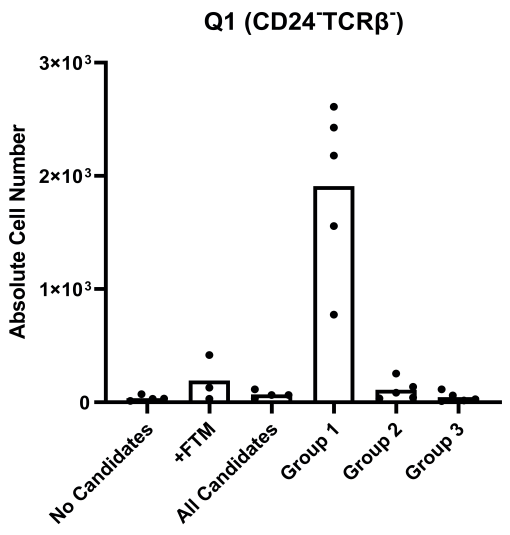

T Cells
Absolute Cell Number
4×10⁴
3×10⁴
2×10⁴
1×10⁴
0
No Candidates
+FTM
All Candidates
Group 1
Group 2
Group 3

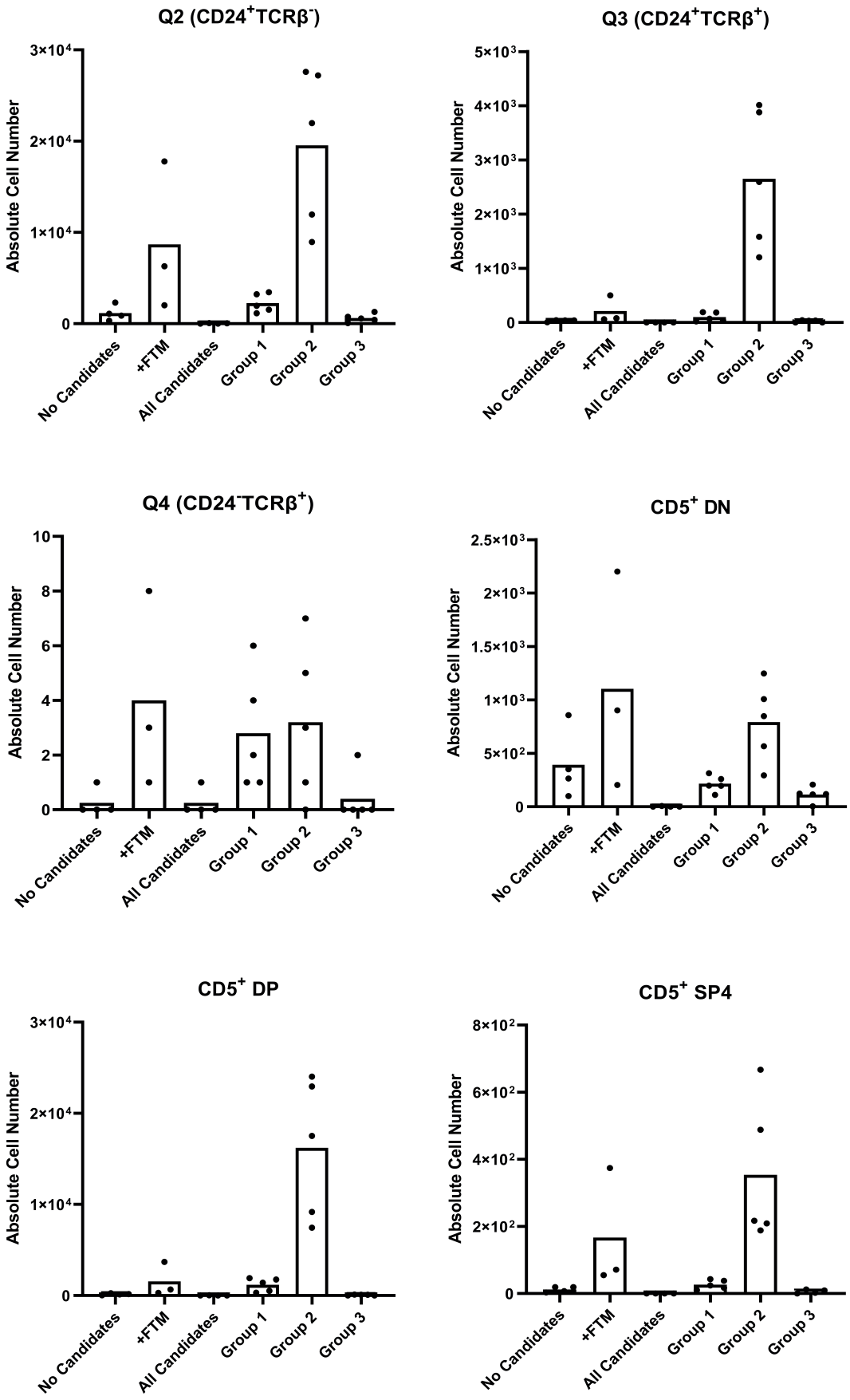

DN
Absolute Cell Number
1.5×10⁴
1×10⁴
5×10³
0
No Candidates
+FTM
All Candidates
Group 1
Group 2
Group 3


DP
Absolute Cell Number
3×10⁴
2×10⁴
1×10⁴
0
No Candidates
+FTM
All Candidates
Group 1
Group 2
Group 3


SP4
Absolute Cell Number
1.5×10³
1×10³
5×10²
0
No Candidates
+FTM
All Candidates
Group 1
Group 2
Group 3


SP8
Absolute Cell Number
4×10³
3×10³
2×10³
1×10³
0
No Candidates
+FTM
All Candidates
Group 1
Group 2
Group 3


Q1 (CD24⁻TCRβ⁻)
Absolute Cell Number
3×10³
2×10³
1×10³
0
No Candidates
+FTM
All Candidates
Group 1
Group 2
Group 3


Q2 (CD24+TCRβ-)
Absolute Cell Number
3×10⁴
2×10⁴
1×10⁴
0
No Candidates
+FTM
All Candidates
Group 1
Group 2
Group 3
Q3 (CD24+TCRβ+)
Absolute Cell Number
5×10³
4×10³
3×10³
2×10³
1×10³
0
No Candidates
+FTM
All Candidates
Group 1
Group 2
Group 3
Q4 (CD24-TCRβ+)
Absolute Cell Number
10
8
6
4
2
0
No Candidates
+FTM
All Candidates
Group 1
Group 2
Group 3
CD5+ DN
Absolute Cell Number
2.5×10³
2×10³
1.5×10³
1×10³
5×10²
0
No Candidates
+FTM
All Candidates
Group 1
Group 2
Group 3
CD5+ DP
Absolute Cell Number
3×10⁴
2×10⁴
1×10⁴
0
No Candidates
+FTM
All Candidates
Group 1
Group 2
Group 3
CD5+ SP4
Absolute Cell Number
8×10²
6×10²
4×10²
2×10²
0
No Candidates
+FTM
All Candidates
Group 1
Group 2
Group 3

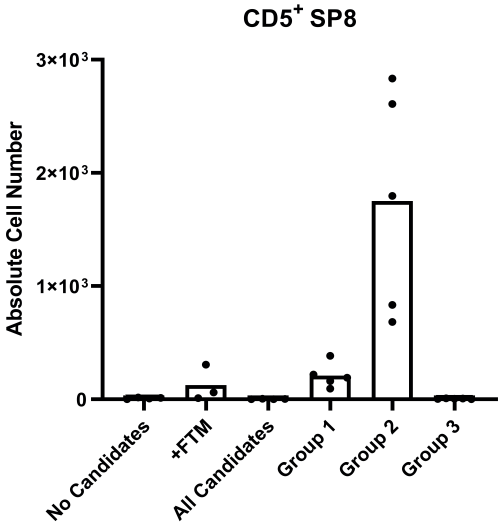


Figure 4.2.18. FTM candidate screen: Absolute cell numbers of thymocytes present in each RTOC condition 14 days *in vitro*. Each datapoint represents one technical replicate. For No Candidates, All Candidates, Group 1, 2 and 3 conditions each RTOC consisted of 600,000 iTEC + 1,000 LMPPs + 100,000 WT MEFs. For + FTM each RTOC consisted of 600,000 iTEC + 1,000 LMPPs + 100,000 WT MEFs + 50,000 Fetal Thymic Mesenchymal (FTM) cells. T Cells ($CD45^+Lin^-$), DN ($CD4^-CD8^-$), DP ($CD4^+CD8^+$), SP4($CD4^+$), SP8 ($CD8^+$), Q1($CD24^-TCR\beta^-$), Q2($CD24^+TCR\beta^-$), Q3($CD24^+TCR\beta^+$), Q4($CD24^-TCR\beta^+$). N=1 experiment.

4.3. Discussion

When establishing the iTEC system, Bredenkamp and colleagues (Bredenkamp *et al*., 2014) reported the transplantation of iTEC alongside other cell types obtained from fetal thymi to recapitulate the native thymus such as fetal thymic mesenchyme (FTM) and DN thymocytes. The requirement of these in the iTEC system was not examined. In this Chapter, I have tested the effect of FTM on thymopoiesis and architecture of grafts, identified ligands produced by FTM that act on TEC and validated some of these in a preliminary experiment *in vitro*. 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.

4.3.A. Fetal thymic mesenchyme in iTEC-RTOC

The results discussed in section 4.1 of this Chapter identified FTM as a beneficial component in iTEC-RTOC with improved progression of DN to DP T cell development in comparison to the counterpart condition lacking it. These findings are in agreement with reports that show FTM plays a role in early thymocyte development specifically in the DN to DP stage. In 1993, Anderson and colleagues found that mesenchymal cells obtained from fetal lung rudiments alongside $MHCII^+$ TEC were essential for T cell development. The need for mesenchymal cells was mapped to the early stages of T cell development, specifically in progression from the DN stage. Anderson and colleagues also reported that the 3T3 fibroblast cell line was able to recapitulate the effect seen from lung mesenchyme on the organ cultures. In 1997 Anderson and colleagues also found that fibroblast action on early thymopoiesis can be specifically pinpointed to the $CD25^+CD44^+$ DN stage, DN2. They stated that fibroblast-associated ECM found in the thymic cortex is the most likely candidate for eliciting the effect seen (Anderson *et al*., 1997). Similarly, Suniara and colleagues reported a block in thymopoiesis at the DN stage of development in E12 lobes cultured *in vitro* following microdissection removal of perithymic mesenchyme (Suniara, Jenkinson and Owen, 2000). In this Chapter, iTEC-RTOC that contained iTEC, DNs and WT MEFs ± FTM were compared. The presence of FTM was found to increase the percentage of DP T cells present in the grafts, in agreement with predictions based on previous reports. However, although fibroblasts of different origins have been reported to elicit similar effects with varying

levels of success regarding thymopoiesis, this was not the case in the iTEC-RTOC system as WT MEFs were included in all conditions and these did not compensate for the absence of FTM.

FTM has also been shown to play a crucial role in thymic organogenesis, TEC proliferation and survival. Experiments carried out by Auerbach (1960) indicated that mesenchyme of different origins could in some cases (lung, submandibular, metanephrogenic mesenchyme) elicit morphogenetic effects on thymic epithelium with varying degrees of success but not in others (limb bud and newborn mouse mesenchyme), suggesting that not all mesenchyme can elicit the effects seen by FTM. Despite FTM having been reported to provide morphogenetic signals for thymic primordia E9 endoderm grafts where pharyngeal mesenchyme was enzymatically removed prior to transplantation under the kidney capsule, grafts were able to give rise to patterned thymi containing a mesenchymal capsule most likely provided by the kidney (Gordon *et al.*, 2004). Jenkinson and colleagues carried out a series of experiments whereby removal of PDGFRα^{+} mesenchyme from fetal thymi was carried out, showing that following transplantation of mesenchyme-stripped primordia, the recovered thymi exhibited full function but were hypoplastic (Jenkinson *et al*., 2007). The group also reported that the recovered grafts displayed infiltration from host kidney-derived mesenchyme, and that this was not fully able to compensate for the lack of FTM, indicating a unique role for FTM in thymic organogenesis and intrathymic niche availability. It has been shown that addition of fibroblasts to thymic organ cultures can help support thymus organogenesis by interacting with FTM (Itoi and Amagai, 1998). However, examination of iTEC-RTOC generated in the absence of WT MEFs was not performed, due to the increased efficiency of reaggregation in the presence of WT MEFs and consequent ease of transfer into transplant recipients.

Patch (*ph/ph*) mice carry a *Ph* locus mutation that spans a large region including the PDGFRα gene which leads to developmental issues that may not be just limited to the PDGFRα mutation (Stephenson et al., 1991). A group studying p*h/ph* mice, that lack PDGFRα^{+} showed that homozygous E12-13 embryos exhibit significantly reduced proliferation of TEC and reduced expression of TEC maturation markers such as MHCII, DLL4 and SCF that could be reversed when introducing FTM into the organ cultures (Itoi *et al*., 2007). Additionally, when investigating the mesenchymal cells of ph/ph mice they found that they cannot form the outer

mesenchymal layers, provide FGFs or migrate into the epithelial cluster of the thymus anlage. That group also stated that FTM was essential for epithelial architecture. Splotch (*Sp*) mutant mice contain a point mutation of *Pax3* resulting in a null allele. *Pax3* is a TF required for NC precursor proliferation and differentiation and therefore $Pax3^{Sp/Sp}$ lack thymic NCCs and the downstream FTM population. A study investigating the effect of this null phenotype on thymic organogenesis from the third pharyngeal pouch (3PP), found NCCs to be involved in 3PP patterning into thymus and parathyroid domains after pouch formation through TF spatial and temporal regulation, but these are not required for initial differentiation of TEC (Griffith *et al*., 2009). In contrast, although Itoi *et al.*, described reduced expression of functional TEC markers on E13 embryos of *ph/ph* the discrepancies between the two reports may be due to the fact that the *Ph* mutation includes other genes.

The results discussed in this Chapter show that the recovered iTEC-RTOC are functional and able to support generation of all major T cell subsets, but that the inclusion of FTM alters thymocyte subset proportions and results in increased graft cellularity. Taken together with the literature discussed above, I conclude that the lack of FTM may result in a lack of intra-RTOC niches to support thymocyte expansion.

Consistent with this, iTEC+FTM-RTOC were indeed able to self-organize *in vivo* and displayed characteristic thymus architecture. As iTEC-RTOC generated without FTM are functional, we conclude that FTM provides factors essential for optimal thymus function, that are not produced by MEFs.

To test whether this may simply reflect support for iTEC themselves, recipients were transplanted with ‘Multi-RTOC’, representing a 3-fold increase in the number of iTEC and number of RTOC per recipient (I note that individual RTOC can fuse upon transplantation to form a single larger structure). Indeed, in transplanted iTEC-MultiRTOC, the proportion of DP thymocytes was higher than in single iTEC-RTOC grafts. iTEC-MultiRTOC also exhibited native thymus architecture with distinct cortical and medullary regions, expressing cTEC and mTEC functional markers such as β5t, AIRE and express MHCII. These data showed that, iTEC-RTOC can support T cell development and develop cortical and medullary compartments in the complete absence of FTM.

4.3.B. FTM candidate selection

As stated above it was hypothesised that FTM provides unique factors to WT MEFs that act on iTEC. MEFs are a mixed mesenchymal population generated by *in vitro* culture of mid-gestation mouse embryos. Therefore, despite both FTM and WT MEFs being fibroblast populations, we would expect them to have distinct transcriptional profiles. Transcriptional profiling has previously investigated the heterogeneity of adult thymic, skin and bone mesenchymal cells (Patenaude and Perreault, 2016). The authors concluded that thymic mesenchymal cells have a distinct transcriptional profile to that of skin and bone, and that gene expression is tailored for interactions with TEC and thymocytes.

In this Chapter the distinct transcription profiles of WT MEFs and FTM were established. In an effort to find factors produced by FTM but not MEFs that act directly on thymic epithelial cells, ligand-receptor analysis was carried out between FTM-TEC and WT MEF-TEC, using bioinformatics analysis of RNA-Seq datasets. This allowed identification of ligands-receptor pairs that had a higher interaction score in FTM-TEC than in MEF-TEC. A list of 25 candidates, that were also commercially available, was identified for experimental validation.

The development of a high-throughput screen for testing the effect of these factors individually, in combination and at varying combinations was not possible due to time limitations. Instead, a preliminary experiment was carried out using iTEC-RTOC, whereby candidates were randomly allocated to one of three groups. In this preliminary experiment, group 2 candidates were found to increase the generation of DPs and SPs, Q3 immature SPs and CD5 expression.

Many studies have reported that some of the candidates identified have a role in the thymus. In Group 2, these can broadly be divided into factors that promote epithelial proliferation and maturation or inhibit thymic involution (Pleiotrophin, Insulin like Growth Factor-1 / Bone Morphogenetic Protein-4, Transforming Growth Factor-β2), promote thymus vascularisation (Vascular Endothelial Growth Factor A), or affect lymphocyte chemoattractant and proliferation (Interleukin-16, Vascular Endothelial Growth Factor A, Transforming Growth Factor-β2, Angiopoietin-like Protein-7, immune response regulation (PF4)). Because these factors were tested in an *in vitro* system, it is highly likely that immune response regulation and vascularisation will not be contributing to the effect seen in cultures on thymopoiesis. Instead, induction of thymocyte and/or TEC proliferation and maturation will most likely be responsible.

Many of these factors may have a concentration threshold after which their presence becomes inhibitory.

Group 1 also contained factors known to positively impact the thymus by reversing thymic involution (Hepatocyte Growth Factor), inducing fetal thymic lobulation and known to support many epithelial cells when supplemented in media (Epidermal Growth Factor), inducing TEC proliferation (Insulin-like Growth Factor 1), contributing to T cell development (Thymic Stromal Lymphopoietin), inducing vascularisation (Placental Growth Factor-1) and assisting in Treg and possibly SP8 intrathymic development (Interleukin-15) (Friend *et al*., 1994; Shinohara and Honjo, 1996; Carrio, Torroella-Kouri and Lopez, 2007; Chu *et al*., 2008; Kang *et al*., 2014; Apert, Romagnoli and van Meerwijk, 2018). Interestingly, IL-7 and IL-15 have a common gamma chain (γc) component of their signalling receptors and IL-7 is known to have blocked DN progression at high doses which form the possibility that IL-15 may be exerting inhibitory effects through signalling in the same manner (Kinter et al., 2008; Elkassar and Gress, 2010). Additionally, mechanisms of action between soluble and membrane-bound IL-15 may be impacting the effect exerted on thymocytes development (Giron-Michel *et al*., 2005). Group 1 also contained two factors known to exhibit inhibitory effects in the thymus such as BMP-2, involved in inhibition of early T cell development, and Midkine, reported to negatively regulate Treg differentiation (Cejalvo *et al*., 2007; Sonobe *et al*., 2012). Despite BMP-2 exerting inhibitory effects on early T cell development (DN to DP differentiation), BMP-2/-4 signalling has been found to be required for thymocyte precursor proliferation (Hager-Theodorides *et al*., 2014). Additionally, as BMP-2 signals through the same receptor as BMP-4 it could also promote *Foxn1*. So, although Group 1 did not elicit positive effects in the iTEC-RTOC cultures, this may be due to BMP-2 known to negatively regulate DN to DP transition of thymocytes.

Finally, Group 3 also contained factors thought to promote various intrathymic processes: thymic development and growth (Bone Morphogenetic Protein-7, Fibroblast Growth Factor-10), generation of naïve T cell subsets (Angiopoietin-1), induction of phagocytosis (Chemerin) (Revest *et al*., 2001; Patenaude and Perreault, 2016; Politikos *et al*., 2017; Miura *et al*., 2020). Additionally, it contained factors known to inhibit lymphocyte chemotaxis (Slit Guidance Ligand 2), thymic growth (Insulin-like Growth Factor Binding Protein-4) and arrest T cell development at the DN stage (Fibroblast Growth Factor-10) (Wu *et al*., 2001; Tsai, Lee and Wu,

2003; Zhou *et al*., 2004). These inhibitory factors may explain the performance of the iTEC-RTOC and the reason why the condition containing all the candidates performed poorly. Testing these candidates in groups without first understanding their individual effect is therefore a confounding factor of these experiments.

In future studies, a medium- or high-throughput *in vitro* culture system that does not rely on large quantities of tissue, once developed, could be employed to test each candidate individually, at varying concentrations and in different combinations. Identifying which of these factors can replace the effect FTM has in the iTEC-RTOC system, could lead to transplantation studies using growth factor beads soaked in these specific factors as have been previously described (Lin, Chen and Slack, 2013; Cruz-Martinez *et al*., 2014; Mohammed and Sweetman, 2016; Uchida and Kanai, 2016).

These candidates could also base the foundation of a series of future experiments with sequential media provision substituted with different factors for different timepoints and requirements of the system. Ideal candidates could be tested for enhancing reprogramming, others for DN T cell development and others for late stages of T cell development. In addition, investigation into alternative groupings based on shared receptor/signalling pathways could be carried out for future experiments.

It is of note that although Group 2 resulted in increased generation of DPs, SPs, Q3 immature SPs and positive selection outperforming iTEC+FTM-RTOC, these RTOC produced 10-fold fewer cells T cells than the native TEC-RTOC condition. The difference in T cell numbers may indicate the requirement of further supplementation with additional factors such as those provided by thymic endothelial cells not investigated in this Chapter.

4.3.C. Considerations regarding experimental design

4.3.C.A. Considerations regarding iTEC-RTOC functionality

It is important to consider variability when examining the outcome of the experiments discussed in this Chapter. The underlying reasons for this variability are as discussed in Chapter 3. Removal of FTM from the system did not reduce the variability in T cell subsets present in the recovered grafts, suggesting that other factors are contributing (see Table 4.1.2). Large variation, however, was also observed in positive control RFTOC samples, suggesting that this is inherent to the

reaggregation system and not necessarily due to iTEC themselves. In order to elucidate the effect that dissociation and reaggregation has on thymopoiesis, differences between RFTOC and FTOC grafts should first be characterised in detail.

Moving forward, it would also be beneficial to carry out further histological experiments to determine the proportion of grafts that are able to establish distinct cortical and medullary regions in both the presence and absence of FTM. For future experiments it would be advantageous to collect iTEC from each transplantation experiment, for RNA-Seq analysis, and combine this information with their thymopoiesis potential and ability to recapitulate thymic architecture. The latter would give valuable insight into identifying iTEC subpopulations which are required for successful experiments.

Lastly, despite iTEC-MultiRTOC partially compensating for the lack of FTM, the extent to which this is achieved has yet to be determined. From histological analysis the lack of FTM does not hinder iTEC-MultiRTOC ability to recapitulate the architecture of the native thymus, however, the extent to which the absence of FTM may impact thymopoiesis has not yet been determined.

4.3.C.B. Considerations regarding FTM candidate screen

Although the experiment described in section 4.2 examines the hypothesis that FTM provides soluble factors to iTEC that improve thymopoiesis present in iTEC-RTOC, the possibility that some of these factors may act directly on thymocytes has not been excluded. In addition to repeating this preliminary experiment, the possibility that these soluble factors may be directly acting on thymocytes needs to be considered. Group 2 factors most likely to act directly on T cells would be IL-16, TGFβ2 and Angiopoietin 7, which are all linked to HSC and T cell proliferation (Langer *et al*., 2004; Lin *et al*., 2015; Tuzova *et al*., 2015). Experiments that could be carried out to test this could include *in vitro* culture of thymocytes in the presence of the chosen factors and the measurement of subsequent proliferative capacity using cell tracker dyes, BrdU incorporation assays or Ki67 expression, in the absence of iTEC. Alternatively, the corresponding iTEC receptors to the substituted factor tested could be deleted by genome engineering or blocked with blocking antibodies prior to generating iTEC-RTOC.

Although Anderson and colleagues reported that FTM could be substituted by a murine fibroblast cell line (3T3), but not soluble factors, in 3T3-conditioned medium

(Anderson, Jenkinson and Moore, 1993), this was not examined in this thesis due to the tumorigenic potential of the NIH/3T3 line which is not compatible with transplantation. Although Anderson and colleagues stated that substitution with soluble factors could not replace the effect elicited by FTM, the preliminary experiment described in section 4.2 suggests this is not the case. The lack of effect noted from substitution with 3T3-conditioned media could have been due to the low concentration of soluble factors present or to the presence of inhibitory factors. The use of defined soluble factors, rather than 3T3-conditioned medium, allows each factor to be tested at a range of concentrations, enabling the generation of a defined culture medium condition compatible with the iTEC system. While soluble factors are convenient for *in vitro* systems, they can also be used *in vivo* by transplanting beads soaked in the soluble factor alongside the cells of interest. When considering this approach, it would need to be examined whether an initial dose of the factor is sufficient or whether continual provision is required, possibly limiting clinical relevance. The possibility that specific factors may be inhibitory at high doses such as those present during initial delivery of factor-soaked beads must also be considered. Utilising an organoid system on a smaller scale than RTOC, such as those generated in microwells, may be useful to upscale experiments and improve the candidate screen in order to test more factors individually and in different combinations to those discussed here. If tissue availability is limited, priority of focus could be given on factors from Group 2 already known to play a role in thymus development or function such as BMP4, TGFβ2 and IGF2.

Despite focus of these experiments being on soluble factor provision it is important to note that cell:cell interaction between FTM and iTEC and/or thymocytes and ECM provided may also be crucial for the effect to take place. Moreover, it may be important for the function of candidates identified that they are in a membrane-bound form to elicit the desired effect. Cytokines are now known to act as membrane proteins or can be released in soluble or extracellular vesicle (exosomes or microvesicles) associated form (Ferro-Novick and Brose, 2013). Therefore, the sole focus of experiments on soluble factors is a limitation to the experimental work described in this Chapter and could be addressed in future experiments. For instance, extracellular vesicle encapsulation of soluble factors could be considered. Lastly a limitation of the ligand-receptor analysis performed is the use of a ligand-receptor interaction database (Ramilowski *et al*., 2015) which remains poorly

annotated. This means that caution must be taken to ensure that any ligand-receptor pairs identified are biologically relevant.

4.4. Conclusions

To conclude, the work described in this Chapter establishes that FTM has an impact on thymopoiesis within iTEC-RTOC, in terms of numbers and percentages of the developing thymocytes present, but is not required for iTEC-RTOC to mediate T cell differentiation per se. It further shows that the requirement for FTM could be at least partially compensated by increasing the number of iTEC and RTOC transplanted per recipient, and that these ‘no-FTM-grafts’ could develop cortical and medullary regions. In addition, this work identified factors produced by FTM that may be responsible for the improved thymopoiesis observed in FTM-containing grafts.