Chapter 3: Characterisation and optimisation of the induced thymic epithelial cell (iTEC) reprogramming system

3. Introduction and aims

In 2014, our laboratory generated a reprogramming system able to generate thymic epithelial cells (TEC) from mouse embryonic fibroblasts (MEFs). Reprogramming was achieved through enforced expression of a single transcription factor crucial in TEC development, *Foxn1,* in MEFs (Bredenkamp *et al*., 2014). These reprogrammed cells will be referred to as induced thymic epithelial cells (iTEC).

In this system, MEFs were derived from transgenic mice that carried an inducible *Foxn1* transgene knocked in to the Rosa26 locus (*Rosa2*$^{CreERt2/CAG\text{-}\ Foxn1\text{-}IRES\text{-}GFP}$ mice; termed iFoxn1 mice herein). iTEC generated from direct reprogramming of iFoxn1 MEFs were able to support T-cell development *in vitro* and upon transplantation with fetal thymic mesenchyme (FTM) and immature thymocytes in reaggregated thymic organ cultures (RTOC), generated an organised and functional thymus (Bredenkamp *et al*., 2014). Although this represented a major advance for the field of thymus biology, further work showed that optimisation of the reprogramming protocol was required. Limitations such as a high degree of variability in reprogramming efficiency between experiments and the reliance on additional fetal thymic cell types for successful engraftment were identified. Of note, the observed increase in variability in reprogramming efficiency is thought to have arisen as a result of an uncharacterised change in the C57BL/6 stock mice at CRM (C57BL/6JSCRM mice), such that, at the start of this study, complete failure of FOXN1-mediated reprogramming to iTEC was often observed using the original reprogramming protocol.

The work described in Chapter 3 therefore aimed to develop robust protocols for (i) MEFs to iTEC reprogramming and (ii) subsequent generation of iTEC-based thymus grafts. Thus, this chapter describes characterisation of iTEC at two different timepoints of reprogramming and establishment of a reliable aggregation period for generating iTEC-based reaggregate thymic organ cultures (iTEC-RTOC) for transplantation. It further describes experiments designed to test whether

enrichment for iTEC that expressed Delta like 4 (DLL4 (a Notch ligand downstream of FOXN1 (Nowell *et al*., 2011; Bredenkamp, Nowell and Blackburn, 2014; Žuklys *et al*., 2016), essential in the thymus for T-cell development (Hozumi et al., 2008; Koch *et al*., 2008)) would enhance the functionality of iTEC-RTOC.

The hypotheses examined in this Chapter were as follows:

(i) Increased duration of exposure to FOXN1, achieved by increasing the time from FOXN1 induction to iTEC harvesting would result in increased numbers of functional GFP^+ iTEC.
(ii) Increased reaggregation time prior to transplantation would improve the recovery rate of iTEC-RTOC grafts.
(iii) DLL4-expression could be used to enrich for functional iTEC, among FOXN1-induced MEFs.

3.1. Establishing iFoxn1 MEFs

In this direct reprogramming system MEFs were established at E13.5 from a mouse line containing two transgenes which are each knocked into the Rosa26 locus: the iFoxn1 (*Rosa26*$^{CAG\text{-}\ Foxn1\text{-}IRES\text{-}GFP}$) and CreERt2 (*Rosa26*^{CreERt2}) transgenes (Hameyer *et al*., 2007), inherited from the parental lines (Figure 3.1.A). Both parental transgenic mouse lines were on the C57BL/6J background. The iFoxn1 transgene is under the control of a CAG promoter followed by a LoxP-flanked STOP codon. *Foxn1* expression levels can be monitored by GFP which is expressed as a separate protein from a bicistronic message, with the *Foxn1* and *eGFP* transgenes separated by an internal ribosomal entry site (IRES). The CreERt2 transgene encodes the CreERt2 fusion protein which is localised in the cytoplasm but, in the presence of tamoxifen or 4-hydroxy tamoxifen (4-OHT) translocates into the nucleus and functions as CRE recombinase, in this case excising the STOP codon in the *Foxn1* transgene. MEFs that have inherited both transgenes from the parents will be referred to as iFoxn1 MEFs from here on. As a negative control, used to control for any off-target effects of 4-OHT or Cre recombinase, we used MEFs that had only inherited the CreERt2 transgene. These are referred as Cre MEFs.

An outline of the method used to generate iTEC is depicted in Figure 3.1.B and described in Chapter 2 (Materials and Methods). By using the standardised protocol described, I aimed to minimise batch-to-batch variation in culturing and reprogramming of the MEFs. The original protocol described used day 4 post 4-

OHT, but the variability observed led me to test a longer reprogramming period to see if this would be advantageous. The iFoxn1 MEFs undergoing reprogramming were harvested at Day 4 or Day 18 post 4-OHT and were then sorted by flow cytometry to obtain the live GFP^+ cell fraction. Cre MEFs did not express GFP and were not sorted (Figure 3.2).

Phase contrast images of Cre and iFoxn1 MEFs at different stages of the reprogramming process are shown in Figure 3.1.C. Native TEC were not cultured in a monolayer for comparison as rapid downregulation of *Foxn1* occurs in a two-dimensional environment altering cell phenotype (Mohtashami and Zúñiga-Pflücker, 2006), but it is reported that they display an epithelial cobblestone like structure (Hiramine *et al*., 1990). Instead, the aim of this experiment was to compare the morphology of iFoxn1 and Cre MEFs at Day 4 and Day 18 for any noticeable qualitative differences. At Day 4 iFoxn1 MEFs still display a morphology typical of fibroblasts, whereas by Day 18, they appear smaller, rounded morphology and form sheet-like structures, more reminiscent of epithelia. From this, I concluded that the longer, 18 day, period of reprogramming resulted in better conversion of MEFs into epithelial cells than the shorter, 4 day, period.

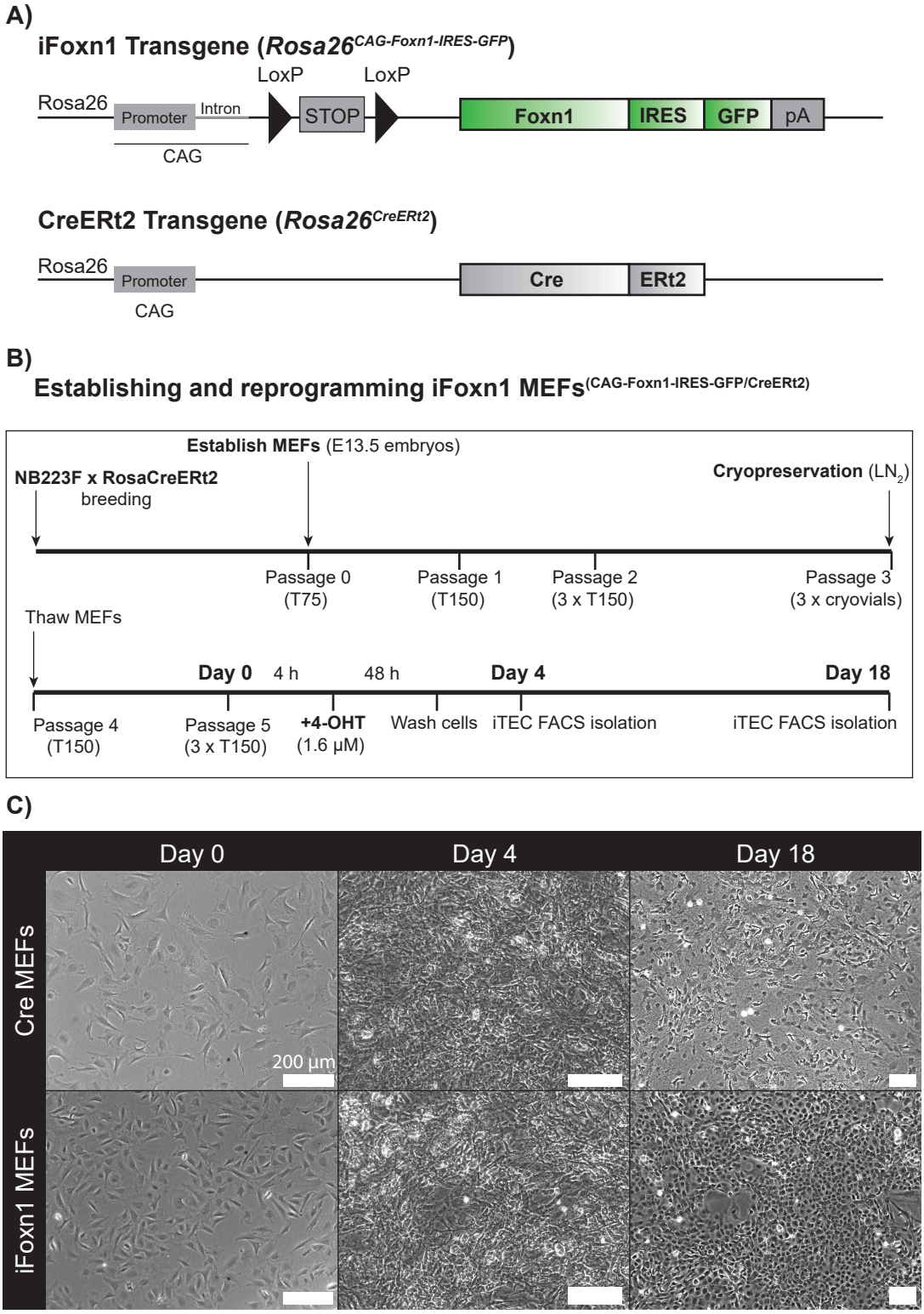


Figure 3.1. Establishing iFoxn1 MEFs. A) Illustration of iFoxn1 MEF transgenes. **B)** Timeline depicting establishment, culture and reprogramming of iFoxn1 MEFs. **C)** Representative phase contrast images of iFoxn1 MEFs and Cre MEFs (negative control) morphology prior (Day 0) and post addition of 4OHT (Day 4 and Day 18 isolation timepoint).

3.2. GFP percentage of cells in Day 4 vs Day 18 iFoxn1 MEFs

Despite epithelial morphology apparent in iFoxn1 MEFs at Day 18 and the apparent absence of this morphology at Day 4 post 4OHT, it remained unknown whether Day 18 would yield a higher proportion of iTEC (GFP^+ iFoxn1 MEFs) than the earlier timepoint. This was tested by flow cytometric analysis of cultures from each timepoint. Representative plots showing the gating strategy used to obtain GFP^+ iFoxn1 MEFs are shown in Figure 3.2.A. The high level of autofluorescence in the GFP channel (diagonal shift) can be explained by the heterogeneity of the MEF population undergoing reprogramming. MEFs and differentiating MEFs will have mixed morphologies and sizes and subsequently autofluorescent profiles. TEC are known to be very autofluorescent, explaining why the autofluorescence profile of cells undergoing reprogramming is different from that of MEFs (see Figure 3.2.A, top right and bottom right panels, respectively). The percentage of GFP^+ iFoxn1 MEFs was determined at Day 4 and Day 18 in four independent experiments (Figure 3.2.B). An unpaired t-test showed a significantly ($p=0.0064$) higher percentage of GFP^+ cells in Day 18 than in Day 4 iFoxn1 MEFs. Note that Day 4 and Day 18 iFoxn1 MEFs were not necessarily obtained from the same embryo, and therefore the unpaired test is appropriate. These data suggested that upregulation of FOXN1, and hence reprogramming, occurs progressively in the cell population and that most MEFs overexpress FOXN1 by the Day 18 timepoint. The GFP^+ fraction of iFoxn1 MEFs at each timepoint will be referred to as Day 4 iTEC or Day 18 iTEC from here on.

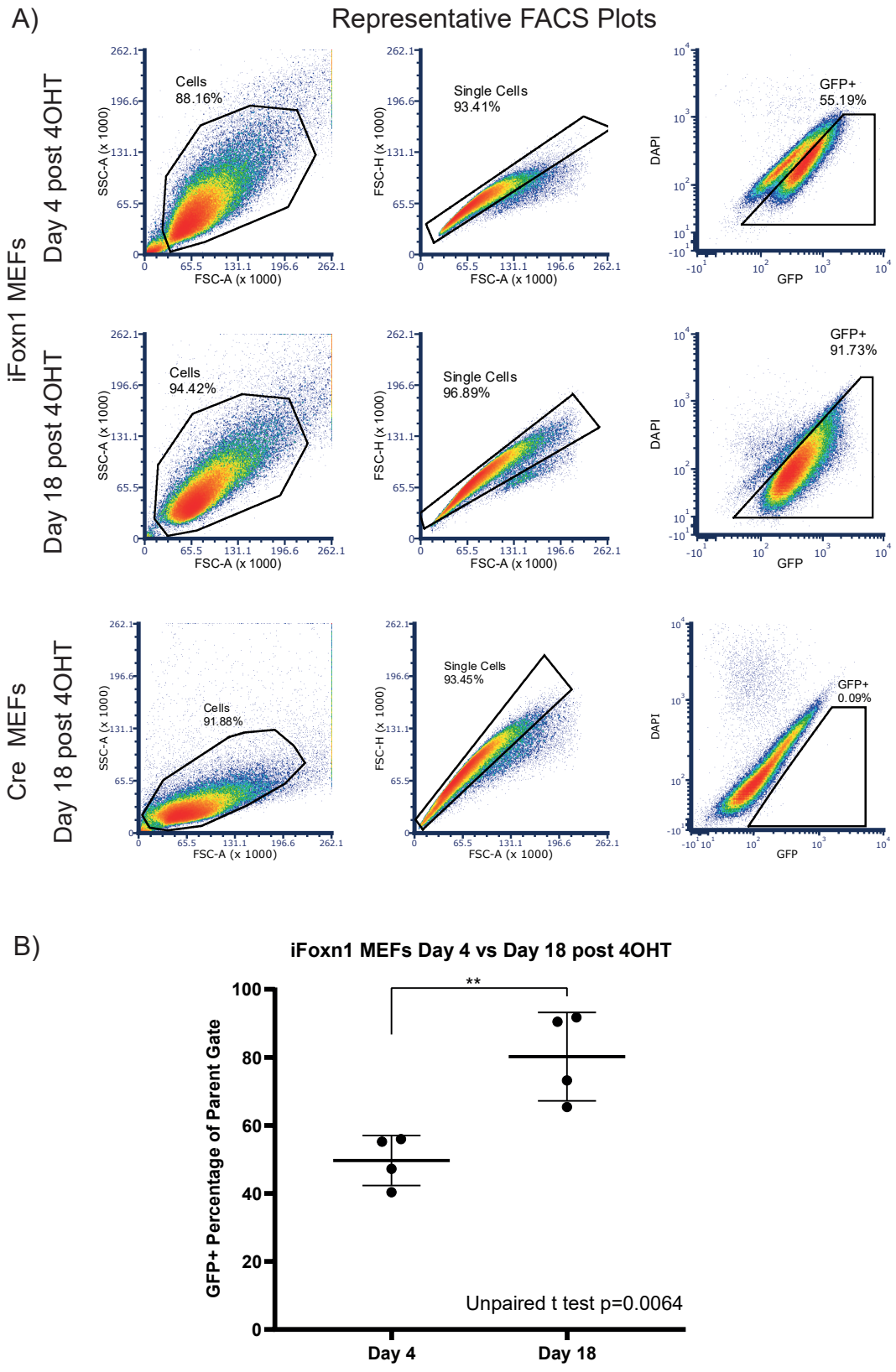


Figure 3.2. GFP% of cells in Day 4 vs Day 18 iFoxn1 MEFs. A) Representative FACS plots of total GFP⁺ cells in iFoxn1 MEFs 4 and 18 days post 4-OHT addition. Negative control Cre MEFs do not express GFP at 18 days post 4-OHT addition. **B)** Collated data of GFP⁺ cell percentages in iFoxn1 MEFs from N=4 independent experiments. Mean ± SD.

3.3. Generating an iTEC derived thymic organoid for transplantation

In order to test whether iTEC functionality recapitulated native TEC, a transplantation assay that tested the ability of iTEC to support T cell differentiation was used. Reaggregate Thymic Organ Culture (RTOC) was used as it allows investigation of T cell development under defined cellular conditions (Anderson, Jenkinson, Moore, 1993). For these experiments, the aim was to generate RTOC in which native TEC were replaced by iTEC. RTOC were generated through the compaction reaggregation of defined ratios of the desired cell types, as previously described (Sheridan *et al*., 2009). iTEC or negative control Cre MEFs were collected and were then combined with fetal thymic mesenchyme (FTM) and DN thymocytes isolated from WT E15.5 thymic lobes by flow cytometric cell sorting (Figure 3.3.A). C57BL/6J E15.5 thymic lobes (syngeneic to iTEC) were microdissected and enzymatically digested prior to antibody staining for flow cytometry. C57BL/6J WT MEFs were also cultured in monolayers and trypsinised for use in RTOC, as carrier cells to bulk out the RTOC. For each individual experiment, more than 50 embryos were required to provide enough FTM for the generation of approximately three RTOC, making this a limiting factor for these experiments.

The desired cell types were combined in defined ratios in a single cell suspension, placed into a sealed non-bevelled p200 yellow pipette tip and compacted at the bottom of the tip by centrifugation at 500 RCF. The resulting cell pellets were then extruded onto isopore membrane filters (hydrophilic polycarbonate membrane, 0.8 µm pore size, Merck, ATTP01300) at the medium gas interface and allowed to reaggregate for the desired length of time. Once successful reaggregation had occurred, RTOC were transplanted under the kidney capsule of syngeneic mice and left for the desired period. This process is illustrated in Figure 3.3.B. Sub-renal capsule transplantation is a well-established technique used to study organogenesis and cytodifferentiation due to the high vascularity of the kidney and increased success rate in comparison to the use of subcutaneous sites (Cunha and Baskin, 2016). This method allows for flexibility in cellular input, aggregation and transplantation period used for experiments.

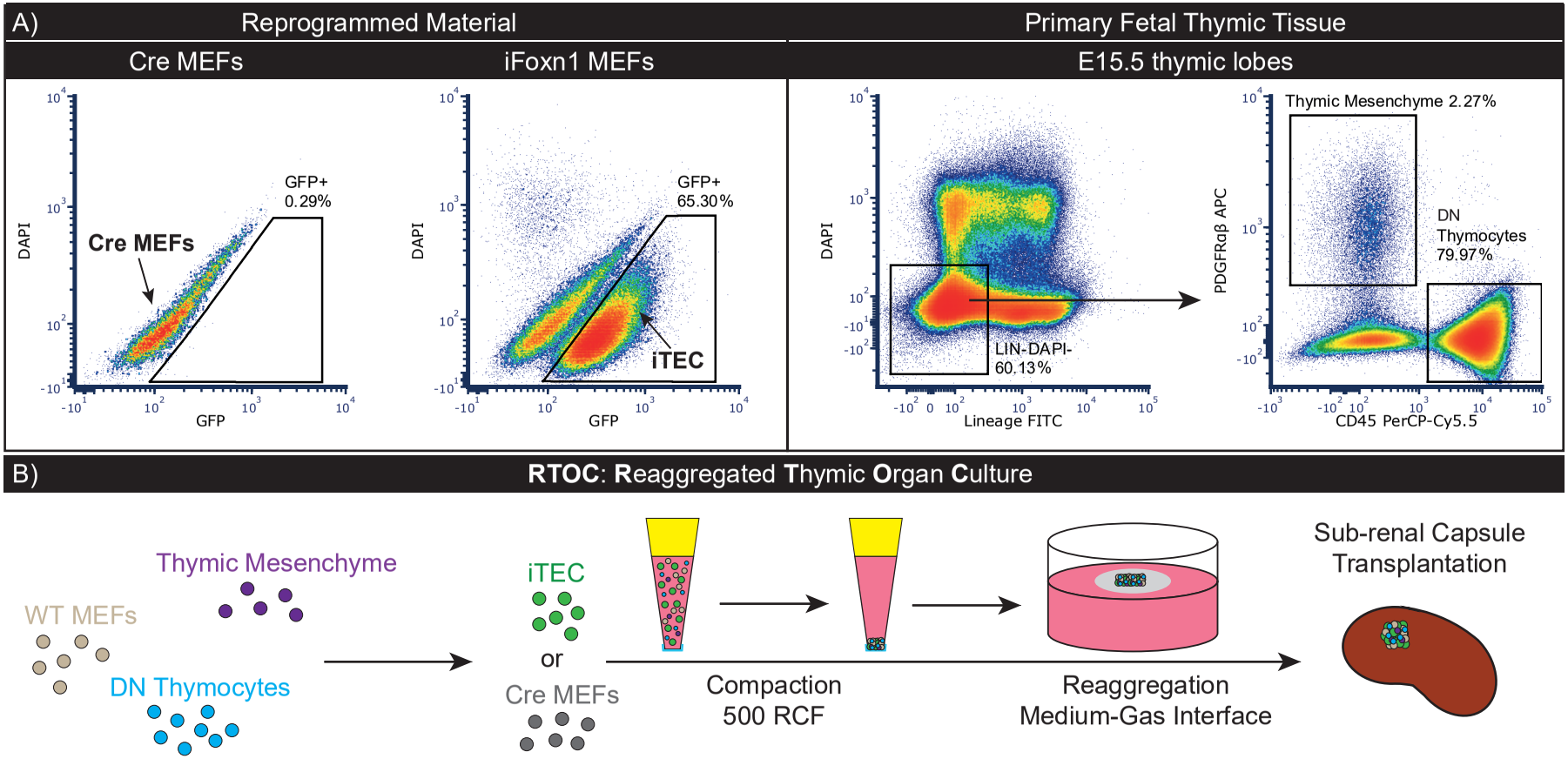


Figure 3.3. Generating a synthetic thymus: Reaggregated Thymic Organ Culture (RTOC). A) Representative FACS plots for obtaining GFP$^+$ iFoxn1 MEFs (left), fetal thymic mesenchyme (FTM) and DN thymocytes from E15.5 thymic lobes (right). iFoxn1 MEFs were sorted from single cells on DAPI$^-$GFP$^+$, FTM on DAPI$^-$LIN$^-$PDGFRαβ$^+$ and DNs on DAPI$^-$LIN$^-$CD45$^+$. Lineage(CD3ε, CD4, CD8, CD11b, CD11c, NK1.1, GR-1, TER119, B220). **B)** Compaction reaggregation of desired cell types. Illustration of cell types used, compaction in p200 non-bevelled tip sealed with parafilm, reaggregation at medium gas interface on millipore membrane and transplantation under the kidney capsule of murine recipients.

3.4. Increasing the reprogramming period of iFoxn1 MEFs and the aggregation period of RTOC increases transplantation success

In the initial stages of this PhD, it was not feasible to recover large enough grafts from transplant recipients for subsequent flow cytometric or histological analysis. This was not due to technical error associated with transplantation, as positive control reaggregated fetal thymic organ culture (RFTOC) returned large grafts (data not shown). I first hypothesised that this was due to too short a reprogramming period for iFoxn1 MEFs (4 days), reasoning that this did not provide sufficient time for iTEC to adopt TEC characteristics. In addition, it was possible that the short RTOC culture period (18h) prior to transplantation did not allow the cells to reaggregate sufficiently well before sub-renal capsule transplantation. An increased reprogramming period of 18 days and an aggregation period of 60 hours were therefore investigated.

Increasing the reprogramming length from four days to eighteen while maintaining the short 18h RTOC culture period produced similar results when grafts were recovered 2 weeks later (Figure 3.4.A and B, respectively). Grafts appeared dispersed under the capsule, rather than localising to one area. RTOC both in the presence or absence of FTM were included in these analyses, due to the limitation of its availability for all experiments, and the presence or absence of FTM did not affect the outcome of these experiments.

In contrast, extending the culture period of the RTOC before transplantation from 18 hours to 60 hours resulted in recovery of grafts with a rounded appearance and compact localisation, that were easier to identify than in previous conditions (Figure 3.4.C). Although qualitative, these results suggested that the longer reaggregation period improved the outcome of iTEC-RTOC transplantation and was carried forward for all future experiments. Day 18 iTEC were also used in all subsequent experiments.

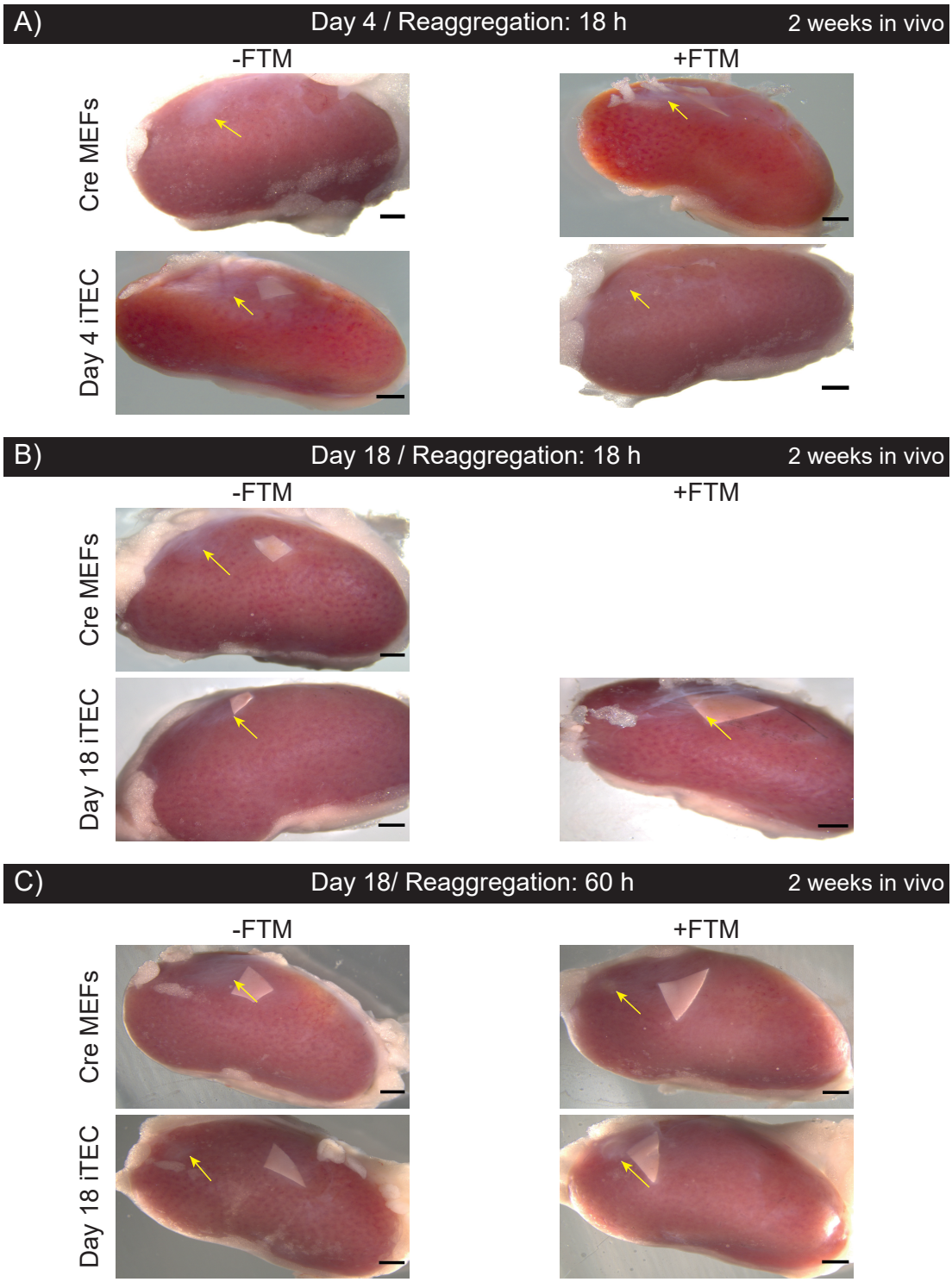


Figure 3.4. Day 18 post 4-OHT iTEC cultured as RTOC for 60 hours produced best grafts. FTM (Fetal Thymic Mesenchyme). **A)** Day 4 iFoxn1 or Cre MEFs were aggregated in RTOC for 18 h prior to sub-renal capsule transplantation. **B)** Day 18 iFoxn1 or Cre MEFs were aggregated in RTOC for 18 h prior to transplantation. **C)** Day 18 iFoxn1 or Cre MEFs were aggregated in RTOC for 60 h prior to transplantation. All RTOC contained 200,000 iFoxn1 or Cre MEFs, 100,000 DN thymocytes, 100,000 WT MEFs and approximately 50,000 fetal thymic mesenchymal cells where indicated (+FTM). Some kidneys contain filter paper to mark graft entry site. Scale bar 1000 μm. Yellow arrows indicate grafted RTOC.

3.5. DLL4 as an additional marker of successfully reprogrammed iFoxn1 MEFs

The iTEC used in all experiments in the above sections were the GFP$^+$ fraction of iFoxn1 MEFs at 18 post days post 4-OHT treatment, based on the assumption that most/all FOXN1-GFP expressing cells would be reprogrammed. In order to further refine the isolation of iTEC, Delta like 4 (DLL4) was included as an additional marker, since DLL4 is directly regulated by FOXN1 in TEC and is also indispensable for T cell development (Koch *et al*., 2008; Nowell *et al*., 2011; Bredenkamp, Nowell and Blackburn, 2014; Žuklys *et al*., 2016).

3.5.A. Gene expression profile of DLL4 enriched iTEC

Notch signalling is essential for specification of haematopoietic progenitor cells (HPCs) to the T cell lineage, DLL4 is the non-redundant ligand required for this *in vivo* (Radtke *et al*., 1999; Han *et al*., 2002; Hozumi *et al*., 2008). TEC-specific *Dll4* deletion leads to a block in T cell development at the DN1 stage of development, with a corresponding increase in intrathymic B cells (Hozumi *et al*., 2008).

Therefore, I tested whether selecting for DLL4$^+$ iTEC among the GFP$^+$ iFoxn1 MEFs and use of these DLL4+ iTEC to form iTEC-RTOC, would result in improved iTEC-RTOC functionality and decrease variability. GFP$^+$ iFoxn1 MEFs will be referred to as iTEC and GFP$^+$DLL4$^+$ iFoxn1 MEFs will be referred to as DLL4 iTEC from here on. The gating strategy used to isolate these cells is shown in Figure 3.5.1.

To interrogate the DLL4$^+$ and GFP$^+$ iTEC populations for phenotypic differences, I first examined expression of key TEC markers in Cre MEFs (negative control), iTEC, DLL4 iTEC and E13.5 thymic epithelial progenitor cells (positive control: TEPCs – PLET1$^+$EpCAM$^+$ cells) (Figure 3.5.2). TEPCs were used as a positive control because gene expression levels in iTEC are similar to those in fetal TEC (Bredenkamp *et al*., 2014), and it is possible that the iTEC population may contain bipotent TEPC.

Real-time quantitative polymerase chain reaction (RT-qPCR) was used to determine mRNA expression levels of selected genes to establish a gene expression profile for each condition. Relative mRNA expression levels were calculated by normalising for three housekeeper genes (*Hmbs, Tbp, Hprt*). For each sample, 100 input cells were used per preparation. Expression of endogenous *Foxn1* was distinguished from total *Foxn1* expression levels (that includes endogenous and transgenic *Foxn1*) using

primers specific for the *Foxn1* 3' UTR, that would only amplify the endogenous gene and not the transgene. This was included as positive autoregulation of *Foxn1* has been demonstrated (Bredenkamp *et al*., 2014). I also tested the expression levels of the Notch ligand Delta-like ligand 1 (*Dll1*); the cytokines *Flt3l* and *Kitl (*Fry et al., 2004; Buono et al., 2016*)*; the chemokines *Cxcl12* (ligand for CXCR4) and *Ccl25* (ligand for CCR9) (Calderón and Boehm, 2011; Žuklys *et al*., 2016); Fibroblast growth factor receptor-2IIIb (*FgfR2IIIb*) (Revest *et al*., 2001; Dooley *et al*., 2007); *Wnt4 (*Wei *et al*., 2015*)* (Wei *et al.*, 2015); *Psmb11* (also known as β5t) (Ripen *et al*., 2011; Uddin *et al*., 2017; Apavaloaei *et al*., 2019). Major histocompatibility complex class II (MHCII) molecules present self-peptides by TEC to T cells that have successfully generated a functional rearranged T cell receptor (TCR) able to recognize self-peptides during central tolerance (Yang *et al*., 2006). The H2 complex that contains the MHCII genes I-A and I-E has a further gene locus name *Eβ* (*MhcII-Eβ*) (Monzón-Casanova *et al*., 2016). Amplification of this region was used to quantify *MhcII* expression. Cathepsin L (*Ctsl*) plays a role in positive selection of CD4 T cells through generation of peptide ligands bound to MHCII molecules on cTECs (Honey *et al.*, 2002). Autoimmune regulatory element (*Aire*) is a transcription factor expressed in mTEC and is required for promiscuous gene expression required for negative selection and central tolerance (Chin *et al*., 2003).

As shown in Figure 3.5.2, the data from this analysis showed a large degree of variation between biological replicates. Alongside the inherent variation of cellular reprogramming, the small number of cells used to quantify mRNA levels of genes may have introduced sampling bias.

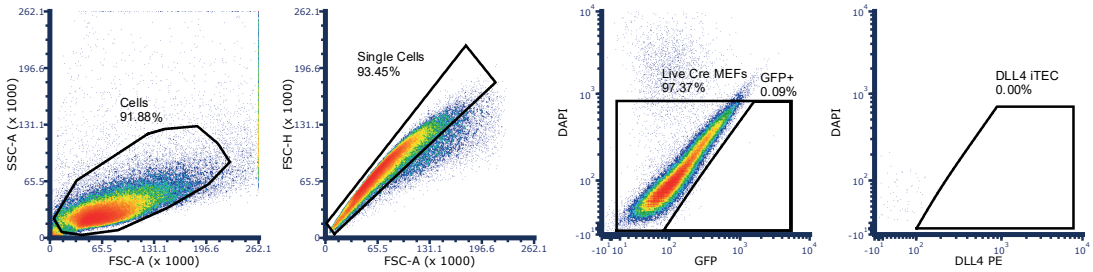
Normal distribution was tested with a Shapiro-Wilk statistical test and significance between samples was inferred with an unpaired t-test for normally distributed data (*Ccl25, Psmb11, Kit-L, Fgfr2IIIb*) and with a Mann-Whitney test for nonparametric data (all remaining genes). As variance was so high and there were only three replicates per gene, no significant differences were found in the nonparametric data.

As expected, total *Foxn1* levels were similar in iTEC and DLL4 iTEC and were approximately 3 times greater than in E13.5 TEPC. *Dll4, Wnt4* and *Psmb11* were not expressed in Cre MEFs, but were expressed at similar levels in both iTEC and DLL4 iTEC, and at higher levels in both iTEC and DLL4 iTEC than in TEPC. Except for *Psmb11*, these differences did not reach statistical significance, due to the high degree of variability between replicates, but nonetheless show a clear trend. *Flt3l,*

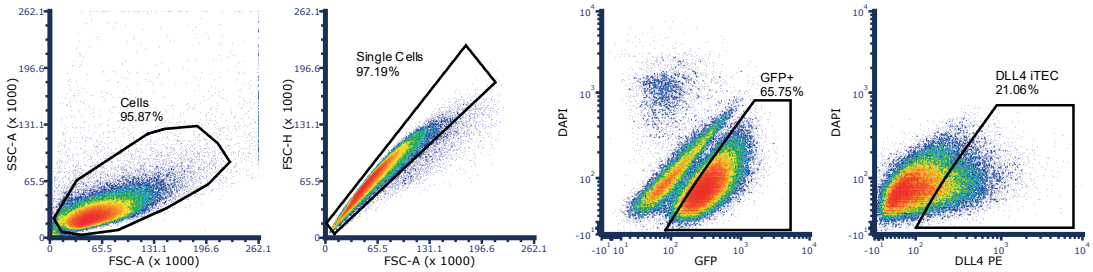
Kitl and *Ccl25* were also expressed only at very low levels or were not expressed in Cre MEFs and were expressed in iTEC and DLL4 iTEC at higher levels than in TEPC. Each of these genes was expressed at higher levels in DLL4 iTEC than in iTEC, with significant differences being observed for *Kitl* and *Ccl25* (*Ccl25* not significant for iTEC versus DLL4 iTEC). Surprisingly, *Cxcl12* was not detected in either iTEC or Dll4 iTEC. Expression of *MhcIIEb* and *Aire* were also not detected, as previously reported, while low level expression of *Fgfr2iiib* was observed but was not significantly different from the level in Cre MEFs. Endogenous *Foxn1* did not appear to be upregulated in iTEC or DLL4 iTEC in comparison to Cre MEFs in contrast to what was previously reported (Bredenkamp *et al*., 2014). *Ctsl* levels in both iTEC and DLL4 iTEC were also very low and only DLL4 iTEC displayed slightly higher levels than Cre MEFs.

Both iTEC and DLL4 iTEC significantly upregulated *Ccl25, Psmb11* and *Kit-L* in comparison to Cre MEFs. TEPCs only upregulated *Ccl25* and *Kit-L* in comparison to Cre MEFs significantly. Only *Kit-L* was significantly upregulated in DLL4^{+} iTEC in comparison to iTEC ($p=0.0142$). It is important to note that although statistical significance was not found for a lot of genes this does not mean that biological relevance is not present. iTEC appear to have successfully reprogrammed due to upregulation of key TEC markers. From looking at trends in the data enriching for a DLL4 iTEC population did not appear to improve phenotype of reprogrammed cells in comparison to GFP whole iTEC.

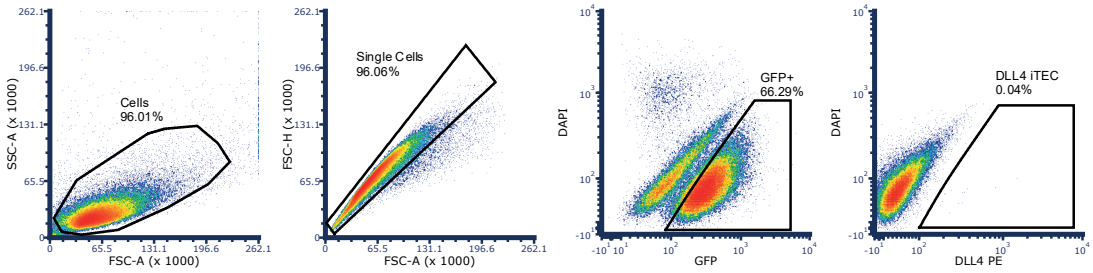
Cre MEFs: Full Stain



iFoxn1 MEFs: Full Stain

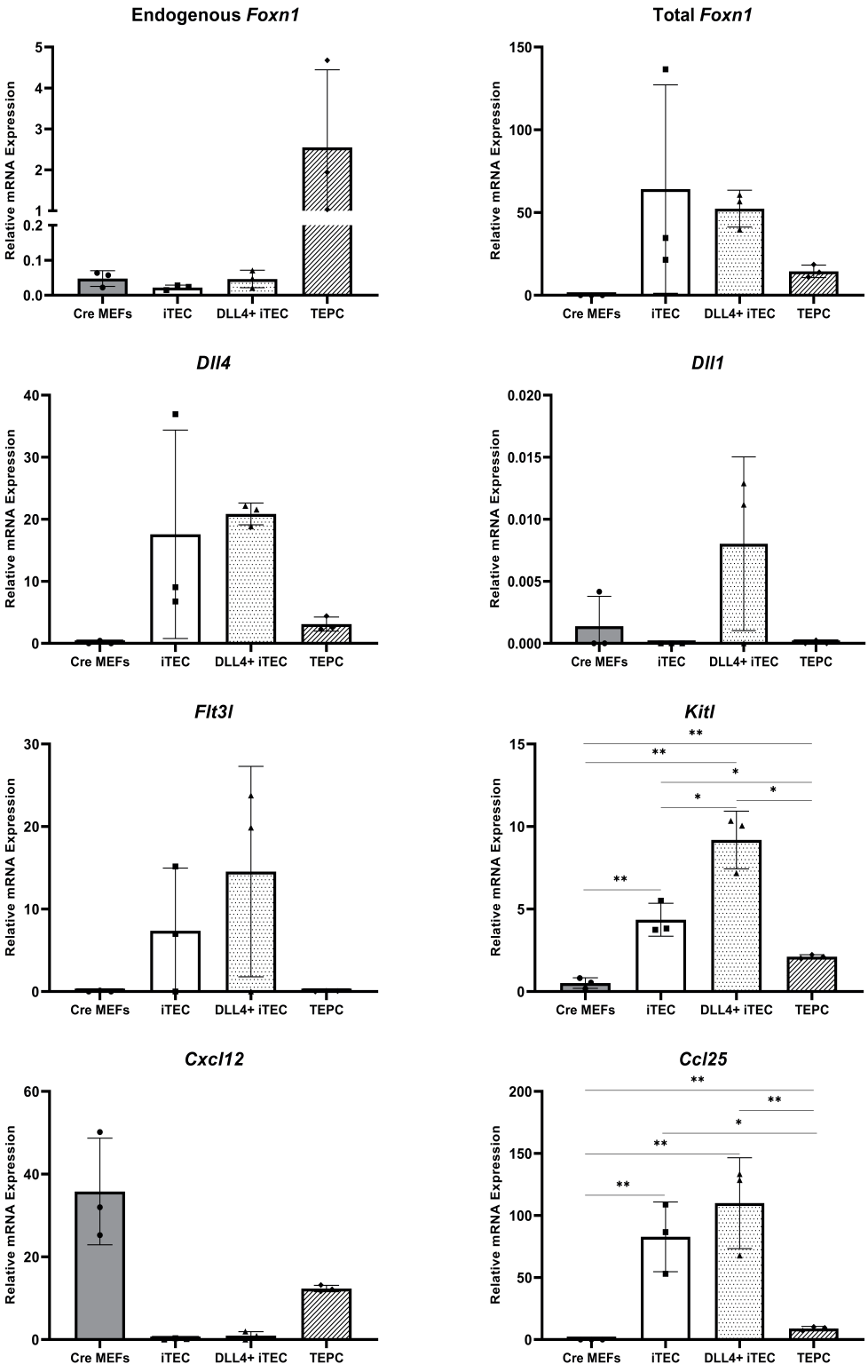


iFoxn1 MEFs: DLL4 isotype



	Cell types & numbers included in each condition						
RTOC Conditions	Cre MEFs	GFP+ iTEC	DLL4+ iTEC	WT MEFs	DN Thymocytes	Thymic Mesenchyme	E15.5 whole dissociated lobes
Cre MEF	200,000			100,000	100,000	50,000	
iTEC		200,000		100,000	100,000	50,000	
DLL4 iTEC			200,000	100,000	100,000	50,000	
RFTOC				200,000			6 lobes

Figure 3.5.1. Gating strategy and experimental conditions used to generate RTOC for sub-renal capsule transplantation. FACS plots are representative for each condition. Numbers in the table refer to cells unless stated otherwise.


Endogenous Foxn1
Total Foxn1
Dll4
Dll1
Flt3l
Kitl
Cxcl12
Ccl25
Relative mRNA Expression
Cre MEFs
iTEC
DLL4+ iTEC
TEPC

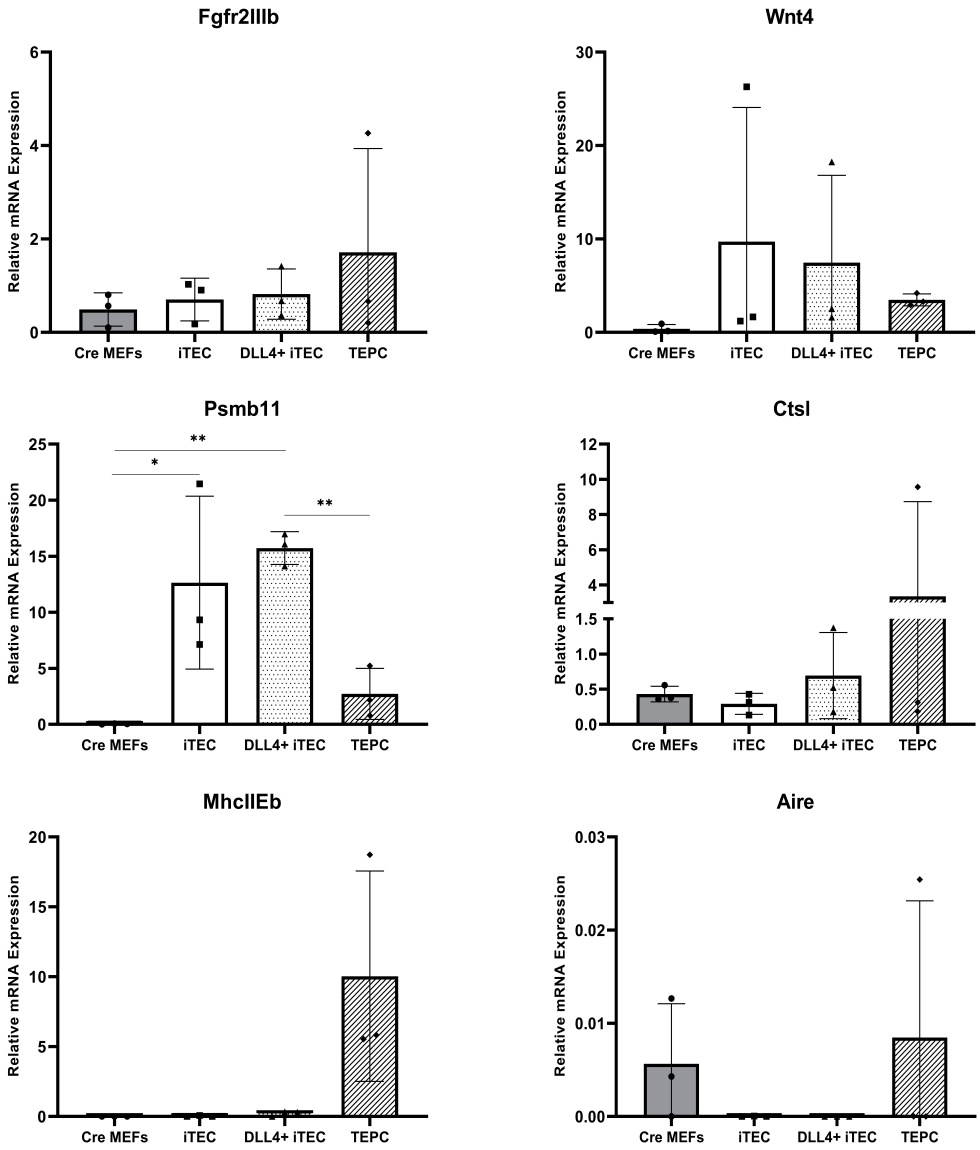


Figure 3.5.2. Gene expression profiles of iTEC and DLL4 enriched iTEC.
Gene expression profiles of key TEC markers in Cre MEFs (negative control), Day 18 GFP^+ whole FACS sorted iTEC (iTEC), GFP^+DLL4^+ iTEC (DLL4+ iTEC) and TEPC (E13.5 PLET1+EpCAM+, positive control) determined by RT-qPCR. Gene expression was normalised to three housekeeper genes (*Hmbs, Tbp, Hprt*) and relative mRNA expression was determined by ΔΔCt values. Where expression of a gene was so low it was not able to provide a Ct value, relative mRNA expression was assigned to 0. Statistical analyses performed were unpaired t-tests or Mann-Whitney tests. * denotes significance with $p \leq 0.05$. Each plot depicts mean±SD of N=3 biological replicates for each condition.

3.5.B. DLL4$^+$ iTEC *in vivo* functionality

Since the above data suggested that there might be some differences between iTEC and DLL4 iTEC, I went on to test whether DLL4 iTEC-RTOC showed improved functionality over standard iTEC-RTOC. Thus RTOC containing iTEC or DLL4 iTEC were transplanted under the kidney capsule of syngeneic mice and left for two weeks, after which grafts were recovered and processed for flow cytometric analysis and histology.

Four conditions were tested. Negative control Cre MEF RTOC, iTEC RTOC, DLL4 iTEC RTOC and a positive control RFTOC (reaggregated fetal thymic organ culture made from 6 whole dissociated E15.5 thymic lobes aggregated with WT MEFs). Details of the cell types and numbers included in each condition are shown in Figure 3.5.1 along with the gating strategy used to obtain Cre MEFs, iTEC and DLL4 iTEC. The gating strategy used for fetal thymic mesenchyme and immature thymocytes is shown in Figure 3.3. As the shift for DLL4$^+$ cells did not result in a distinct separation of the positive and negative populations an isotype control was used to gate the cells. E15.5 DN thymocytes were selected after excluding mature T cells, natural killer cells, SP or DP T cells, B cells, dendritic cells, macrophages, neutrophils, monocytes, granulocytes and red blood cells using a lineage cocktail comprising the following markers: CD3ε, CD4, CD8, CD11b, CD11c, GR-1, NK1.1, TER119, B220 all conjugated to FITC.

Functional analysis of iTEC and their ability to support progression through thymopoiesis was quantified using flow cytometry. The stages of thymopoiesis investigated were DN (CD4$^-$CD8$^-$), DP (CD4$^+$CD8$^+$), SP4 (CD4$^+$), SP8 (CD8$^+$) and total CD3ε$^+$TCRβ$^+$ T cells. Analysis was performed on a BD 5 LSR Fortessa^{TM} that allowed for accumulation of cell percentage data, as cell number were too low to accurately count using a haemocytometer. Grafts were recovered after 2 weeks, and single cell suspensions of thymocytes were obtained by mechanical dissociation and filtering through a 40 μm cell strainer. Three independent experiments were carried out.

Representative FACS plots and the gating strategy used for each stage of thymopoiesis are shown in Figure 3.5.3, 4, 5, 6 for conditions RFTOC, Cre MEF RTOC, iTEC RTOC and DLL4 iTEC RTOC. Figure 3.5.7 depicts CD4 and CD8 T cell plots for each replicate of each condition to display variability between grafts.

Some variability was observed between biological replicates for each condition, as shown in (Figure 3.5.7 and Table 3.1). The percentage of DP cells was used to assess the success rate of grafts in each condition, as this population is found exclusively in the thymus and an indication of whether grafts were capable of the initial stage of thymopoiesis: T-cell lineage specification. It is also important to note that as a lineage panel was not included it is possible for $CD45^+$ cells with low levels of CD4 to also include B cells, dendritic cells or macrophages (Wood, Warner and Warnke, 1983).

Normal distribution was tested with a Shapiro-Wilk statistical test and significance between samples was inferred with an unpaired t-test for normally distributed data. It was found that iTEC-RTOC had significantly higher percentages of $CD3\varepsilon^+TCR\beta^+$, DN and DP T cells than Cre MEF-RTOC ($p=0.0354$, $p=0.0038$, $p<0.0001$). $DLL4^+$ iTEC-RTOC had significantly higher levels of $CD45^+$ cells and $CD3\varepsilon^+TCR\beta^+$ than Cre MEF-RTOC ($p=0.0075$, $p=0.0366$). No significant differences were detected between iTEC-RTOC and DLL4+ iTEC-RTOC.

Condition		CD45$^+$ cells	DN	DP	SP4	SP8	CD3ε$^+$TCRβ$^+$ T cells
Cre MEF	Rep A	11.14	82.4	0	14	3.6	14
	Rep B	5.28	50	0	50	0	43.75
	Rep C	2.02	58.01	1.66	17.68	22.65	37.57
	Mean	**6.15± 3.77**	**63.47± 13.78**	**0.55± 0.78**	**27.23± 16.17**	**8.75± 9.94**	**31.77± 12.82**
iTEC	Rep A	75.31	4.58	92.16	2.93	0.33	2.24
	Rep B	9.18	4.2	93.54	1.2	1.07	2.2
	Rep C	80.85	5.37	86.64	4.84	3.15	5.38
	Mean	**55.11± 32.56**	**4.72± 0.49**	**90.78± 2.98**	**2.99± 1.49**	**1.52± 1.19**	**3.27± 1.49**
DLL4 iTEC	Rep A	56.38	85.69	9.17	3.46	1.68	4.53
	Rep B	57.28	14.54	81.32	1.37	2.77	1.34
	Rep C	93.71	0.8	94.38	3.64	1.17	4.79
	Mean	**69.12± 17.39**	**33.68± 37.20**	**61.62± 37.47**	**2.82± 1.03**	**1.87±0.67**	**3.55±1.57**
RFTOC	Rep A	94.77	0.95	73	13.59	12.46	25.92
	Rep B	95.91	13.7	44.36	27.67	14.27	36.39
	Rep C	91.62	1.76	94.12	2.54	1.58	2.62
	Mean	**94.1± 1.81**	**5.47± 5.83**	**70.49± 20.39**	**14.6± 10.28**	**9.44±5.60**	**21.64±14.11**

Table 3.1. Cell percentages of parent gate for T cell populations in recovered grafts. Detailed account of cell percentages of populations for three biological replicates per condition along with mean ± SD.

The iTEC and DLL4 iTEC RTOC performed similarly in this assay, each supported thymocytes developmental progression from DN into the DP and subsequently SP4 and SP8 stages, with a slight SP4 bias (Figure 3.5.5, 6, 7). Visual inspection also indicated much greater cell numbers in the iTEC, DLL4 iTEC and RFTOC conditions compared to Cre MEFs (see cell density plots). The T cells in the Cre MEFs grafts are likely to represent recirculating peripheral T cells rather than cells produced by Cre MEFs-mediated thymopoiesis given that only SP cells and no DP cells were found within these grafts. In all grafts, most $CD3\varepsilon^{+}TCR\beta^{+}$ T cells were SP4 or SP8 cells as expected. iTEC samples appeared similar to the positive control RFTOC, apart from the fact that the positive control contained a higher proportion of SP4 and SP8 cells. This may suggest accumulation of DP cells in the iTEC condition, with poor progression into SP4 or SP8, or might suggest reduced proliferation of SP4 and SP8 cells once produced.

In the negative control Cre MEF RTOC, very few cells were present (see cell density plots), despite certain populations appearing high in percentage terms (Figure 3.5.4). Furthermore, the percentage of $CD45^{+}$ cells in the Cre MEFs grafts did not exceed 11% whereas in iTEC or DLL4 iTEC grafts only one biological replicate displaying less than 56% (Rep B, iTEC, 9%; Table 3.1). Despite the low percentage of CD45+ cells in iTEC RTOC Rep B, this was not regarded as a technical failure as 93.54% of those cells were DP, indicating functionality of the iTEC (Table 3.1). It is of note that one biological replicate of DLL4 iTEC RTOC was excluded as a technical failure, as it did not support progression into the DP stages of T cell development (data not shown).

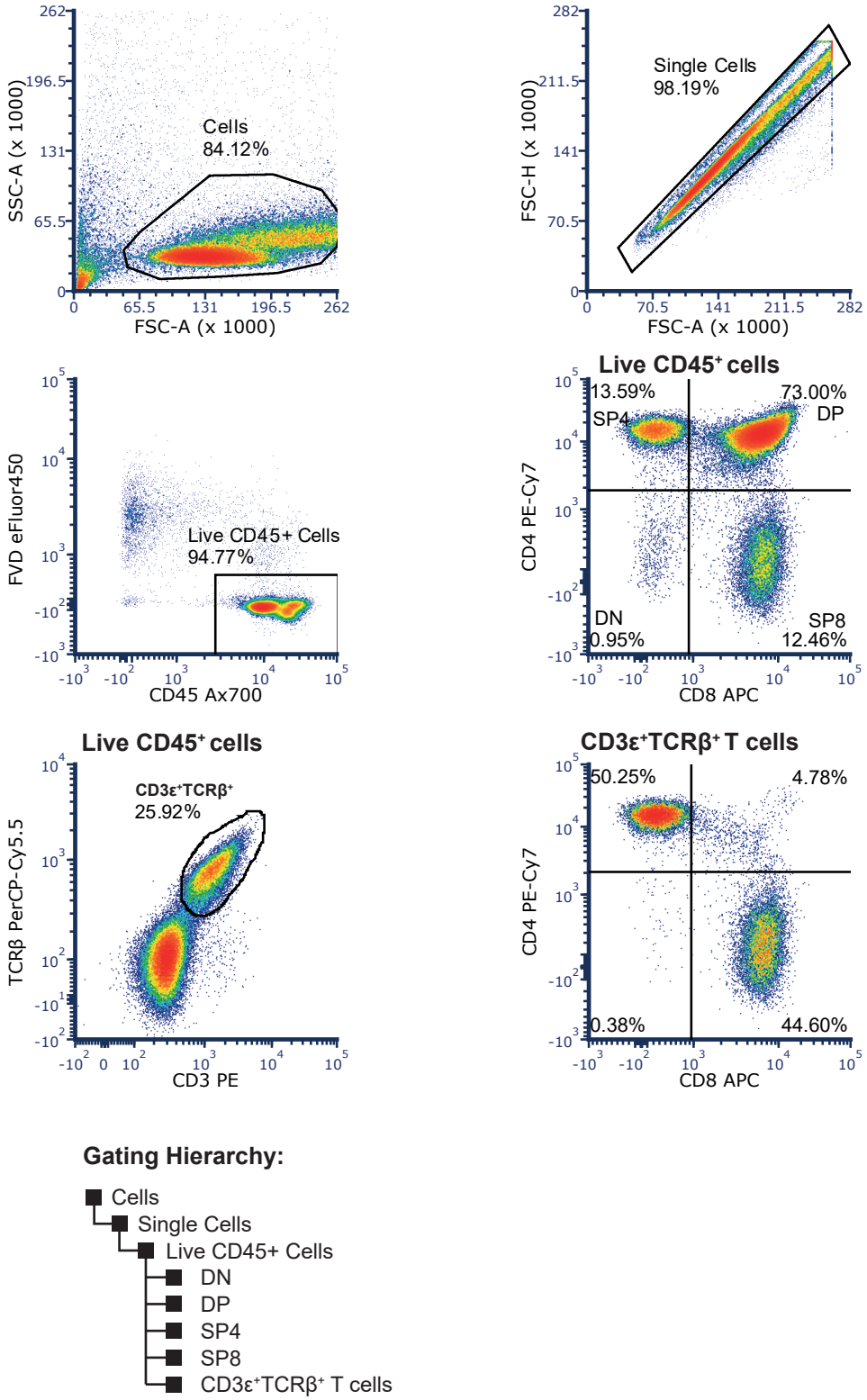


Figure 3.5.3. RFTOC functionality 2 weeks *in vivo.* Representative FACS plots for positive control RFTOC 2 weeks post transplantation. Transplant recipients received one RFTOC consisting of 6 whole E15.5 dissociated fetal thymic lobes +200,000 WT MEFs.

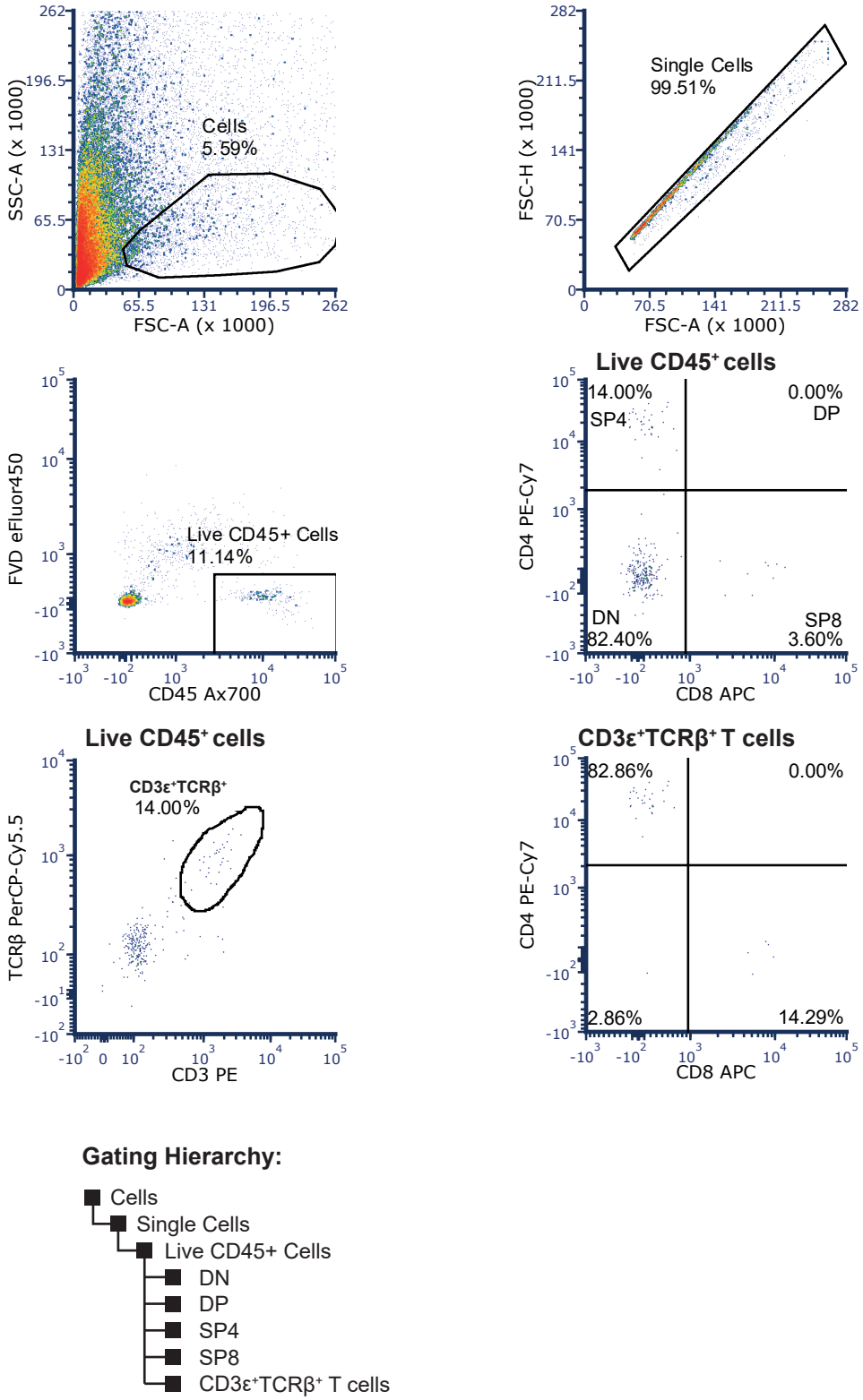


Figure 3.5.4. Cre MEF RTOC functionality 2 weeks *in vivo*. Representative FACS plots for Cre MEF RTOC 2 weeks post transplantation. Transplant recipients received one RTOC consisting of 200,000 Cre MEFs + 100,000 DN Thymocytes +100,000 WT MEFs + 50,000 Fetal Thymic Mesenchyme cells.

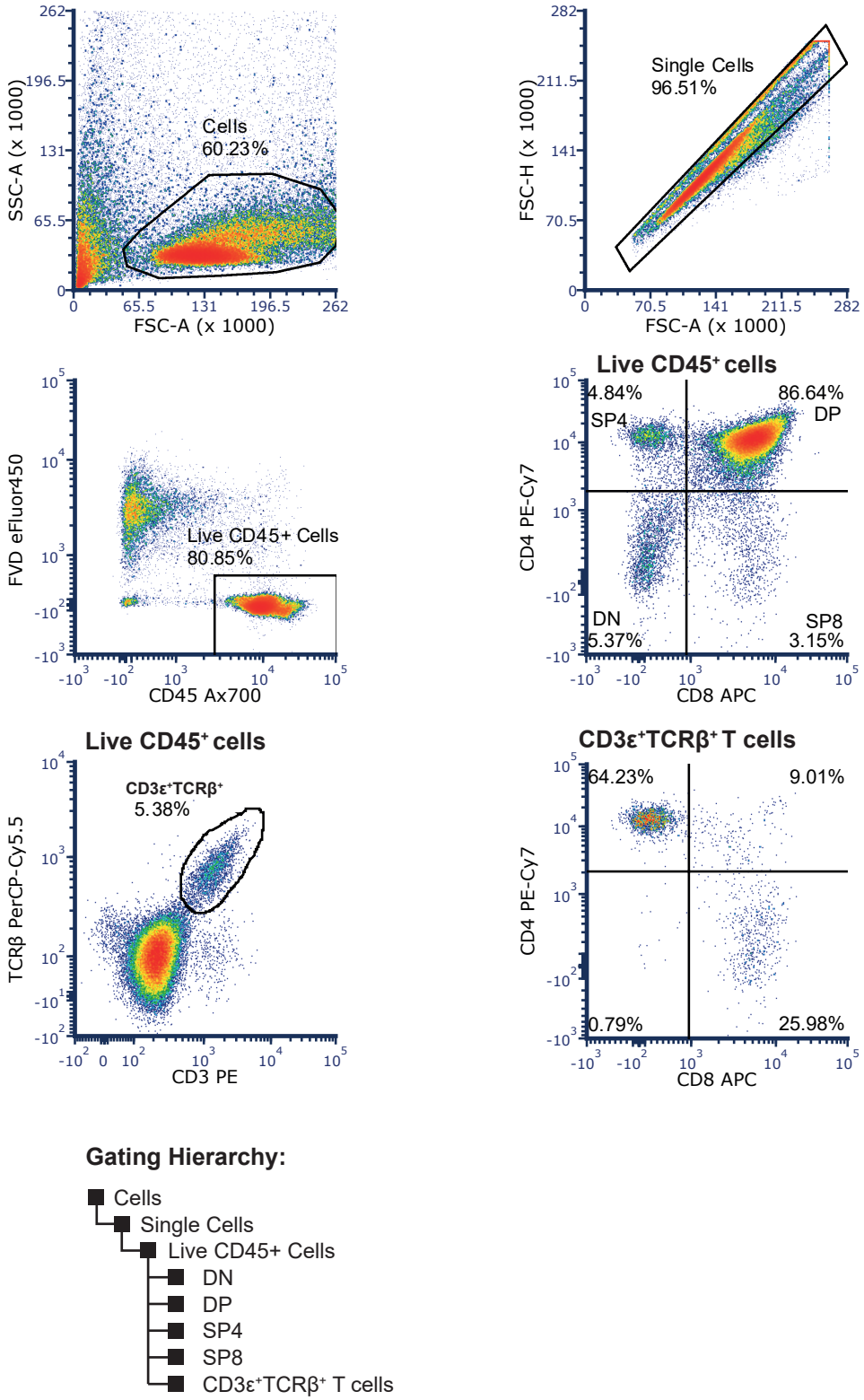


Figure 3.5.5. iTEC RTOC functionality 2 weeks *in vivo.* Representative FACS plots for iTEC RTOC 2 weeks post transplantation. Transplant recipients received one RTOC consisting of 200,000 GFP+ iTEC + 100,000 DN Thymocytes +100,000 WTMEFs + 50,000 Fetal Thymic Mesenchyme cells.

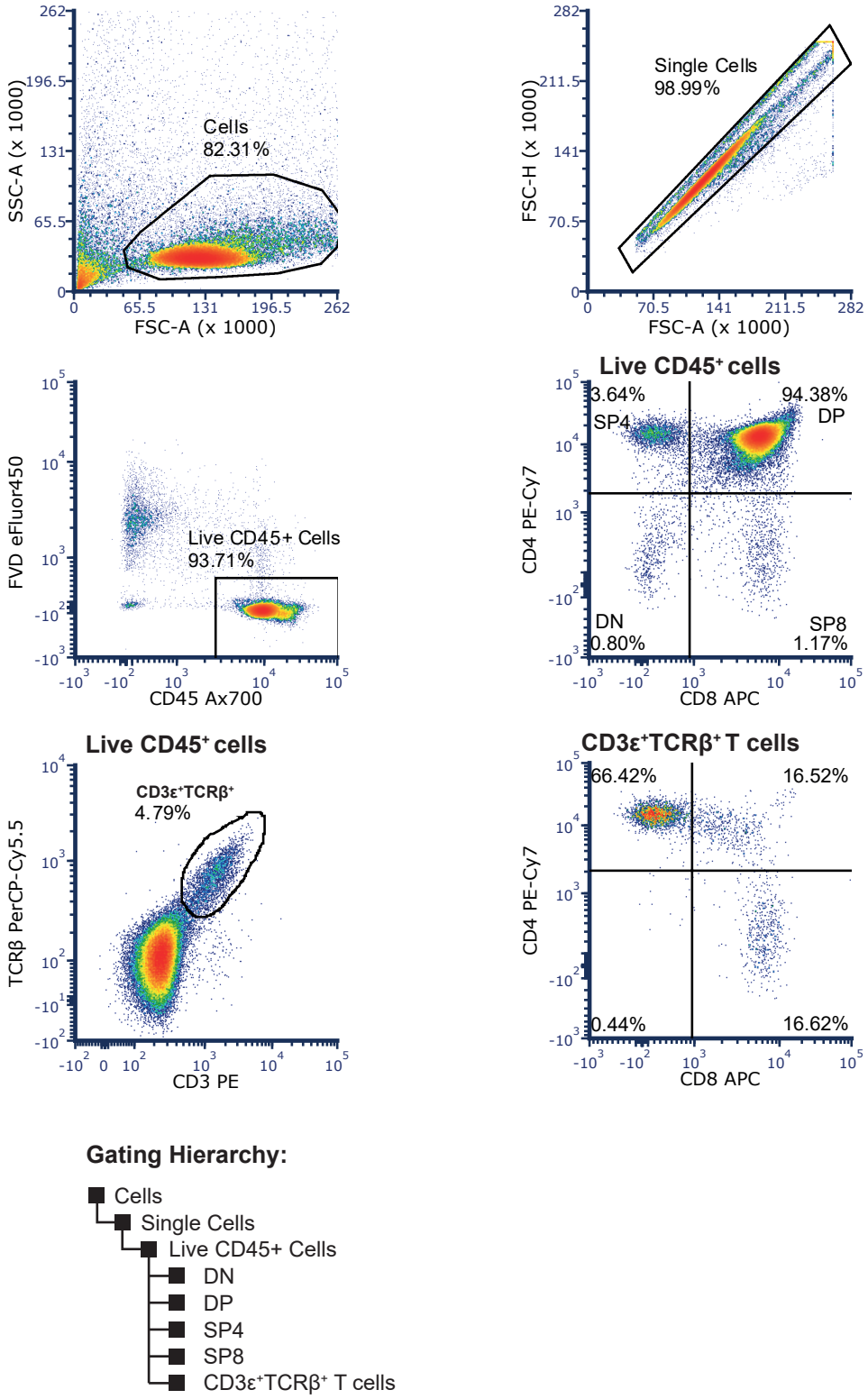


Figure 3.5.6. DLL4 iTEC RTOC functionality 2 weeks *in vivo.* Representative FACS plots for DLL4 iTEC RTOC 2 weeks post transplantation. Transplant recipients received one RTOC consisting of 200,000 GFP+DLL4+ iTEC + 100,000 DN Thymocytes +100,000 WT MEFs + 50,000 Fetal Thymic Mesenchyme cells.

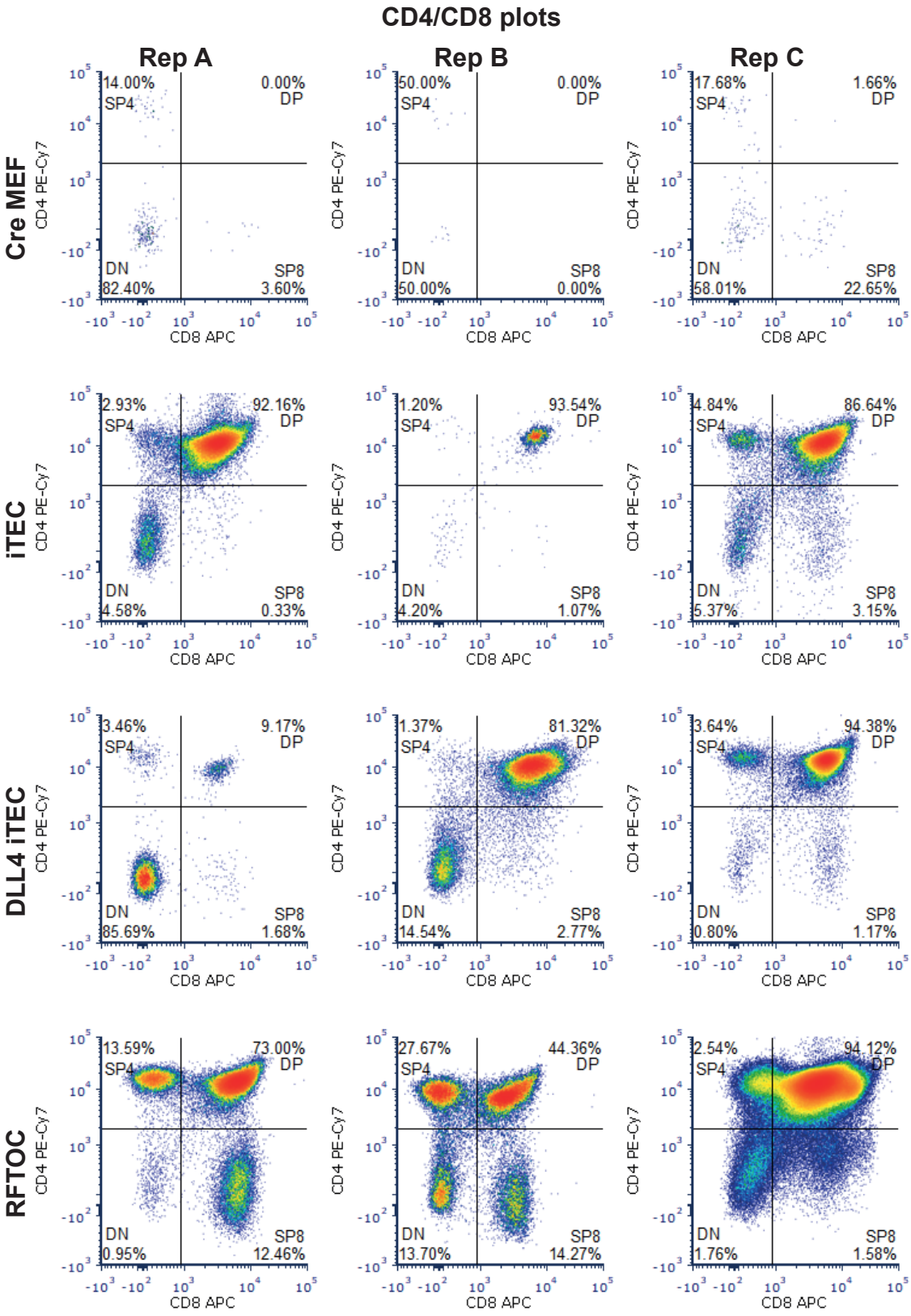


Figure 3.5.7. CD4/CD8 FACS plots for each experiment. Collated FACS plots of CD4 and CD8 T cell profiles of each RTOC condition for each of the three independent experiments.

3.5.C Graft histology in iTEC vs DLL4 iTEC grafts

Following evaluation of the capacity of the iTEC and DLL4 iTEC to support thymopoiesis, I examined the phenotype and architecture of these grafts via brightfield imaging, hematoxylin & eosin (H&E) staining and immunohistochemistry (IHC) of the recovered grafts. Two biological replicates per condition were carried out for this experiment. The conditions examined were as described in Figure 3.5.1. The recovered Cre MEF RTOC grafts were too small to undergo histological analysis in this set of experiments.

Brightfield and H&E images of the recovered grafts and sections are shown in Figure 3.5.8 and Figure 3.5.9, respectively. Low power images showing the majority of the graft, plus representative higher power images showing regions of interest (ROI), are included to indicate the presence or absence of cortical and medullary organisation. In the thymus cortical regions are darkly stained with H&E due to the dense packing of developing thymocytes, which are small with little cytoplasm (Hale *et al*., 2020). The medullary regions appear pinker as thymocytes are less densely packed in the medulla (Hale *et al*., 2020).

For Rep A of this experiment, grafts were removed from the kidney (as it is innately autofluorescent) cryosectioned and processed for staining. In Figure 3.5.8.B. H&E staining of approximately the midsection of the graft can be seen in the top panel showing an overview of the graft and more detail can be seen in the bottom panel where an ROI was selected. In Rep A it is apparent that iTEC and DLL4 iTEC are bipotent and able to self-organise upon transplantation into distinct cortical and medullary regions like the positive control RFTOC as indicated with c and m. iTEC and DLL4 iTEC grafts are smaller than RFTOC. It is also noteworthy that cortical and medullary regions appear more distinct in the iTEC graft in comparison to the DLL4 iTEC graft. Interestingly DLL4 is associated with cTEC lineage but it is apparent from this data that DLL4 iTEC are also able to give rise to the mTEC lineage (Fiorini *et al*., 2008; Meireles *et al*., 2017) although this remains to be confirmed with functional markers.

Replicate B of this experiment produced only small grafts. It was possible to obtain histological and IHC data from these grafts, which reinforced this conclusion (Figure 3.5.9.A). These show the absence of a typical thymus structure, with few T cells present and no cortex/medulla structure.

I also analysed the above grafts by IHC for cytokeratin 8 (K8: green), an epithelial cell marker associated with cTEC (Klug *et al*., 1998, 2002; Danzl *et al*., 2014), Ulex Europaeus Agglutinin-1 (UEA1: magenta) a lectin associated with binding to mTEC (Klug *et al*., 1998; Kuraguchi *et al*., 2006; Lomada *et al*., 2007), and CD45 (cyan) that marks all cells of haematopoietic origin (Altin and Sloan, 1997). As with H&E data (Figures 3.5.8 and 9) Rep A showed distinct medullary regions whereas Rep B did not. For Rep A, the iTEC graft displayed much larger medullary regions than the DLL4 iTEC graft (representative medullary regions are outlined in white in Figure 3.5.10). In contrast, in Rep B no distinct demarcated cortical and medullary regions were present in either condition, but instead displayed spotty staining of $UEA1^{+}$ cells throughout $K8^{+}$ cells. Rep A images were obtained from cryosections and imaged on a Zeiss observer widefield microscope. Rep B images were obtained from wholemount samples, imaged on an SP8 Leica confocal microscope, and represent a flattened z-stack based on maximum intensity of each channel, however prior to this each slice was viewed separately and no organisation was present. Images were edited on ImageJ and the gamma value was altered for the UEA1 channel from 1 to 0.5 to intensify brightness in cells expressing medium levels of UEA1 without oversaturating cells expressing high levels so that they were easier to view. An isotype control with the same settings is included to show that this manipulation did not display non-specific signals.

Consistent with the flow cytometry results presented above, this analysis revealed large variation between biological replicates, in this case in terms of the organisation of the grafts. Although additional replicates would be needed to quantify this variability, these were not pursued as material was limiting and other work was prioritised (see Chapter 4).

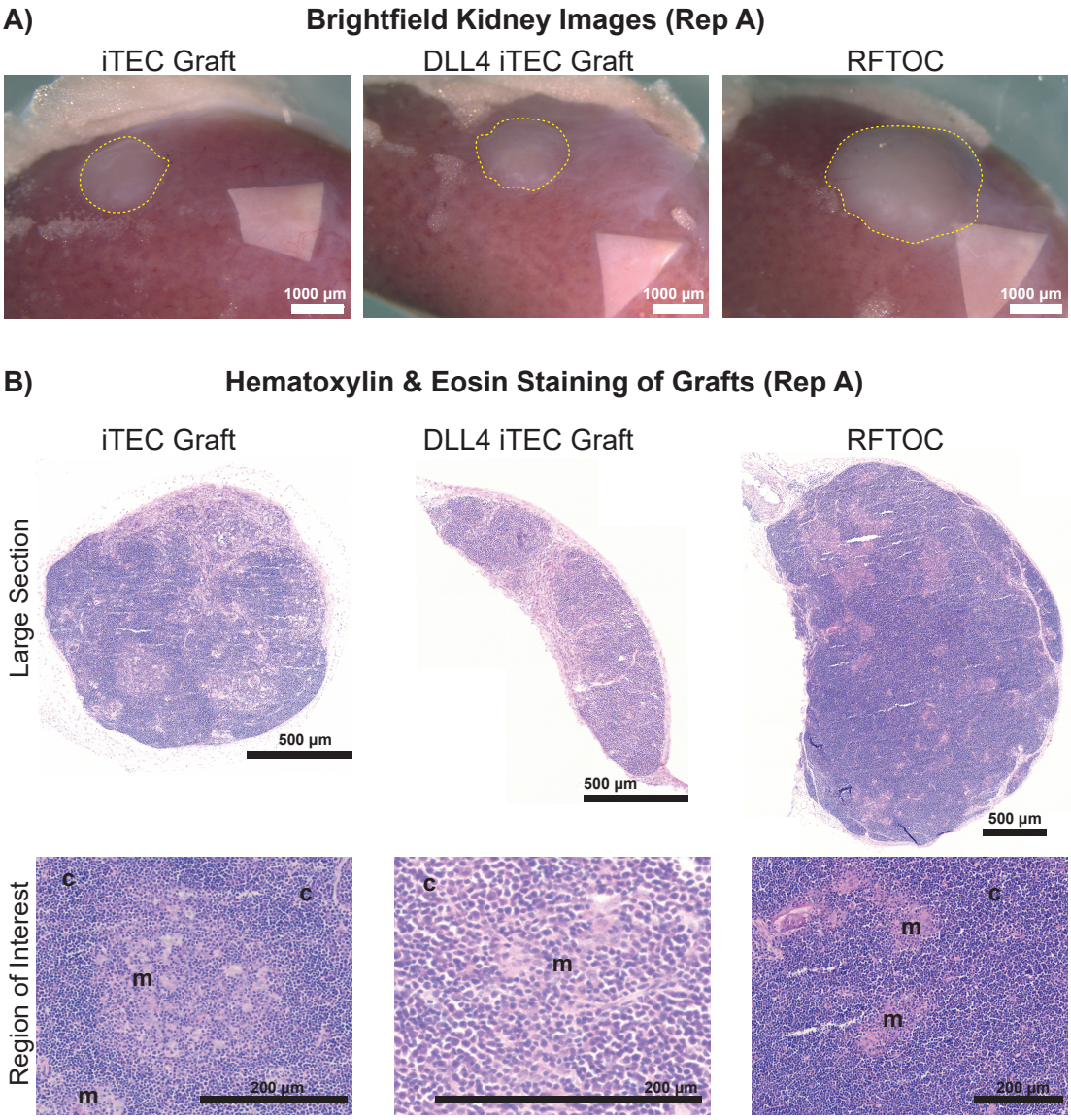


Figure 3.5.8. Histology of recovered grafts two weeks in vivo in Rep A. A) Brightfield images of kidneys used for iTEC RTOC, DLL4 iTEC RTOC and RFTOC transplantation in Rep A. Grafts are outlined in yellow lines. **B)** Haematoxylin and eosin (H&E) staining of sectioned recovered grafts. In the top panel the whole area of the isolated sectioned graft is depicted. In the bottom, regions of interest were focused on to show 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. Data for Cre MEFs were not shown as grafts were too small to isolate from under the kidney capsule and undergo histological processing in the same manner as above. This experiment was repeated once more to a total of N=2 biological replicates for histological analysis.

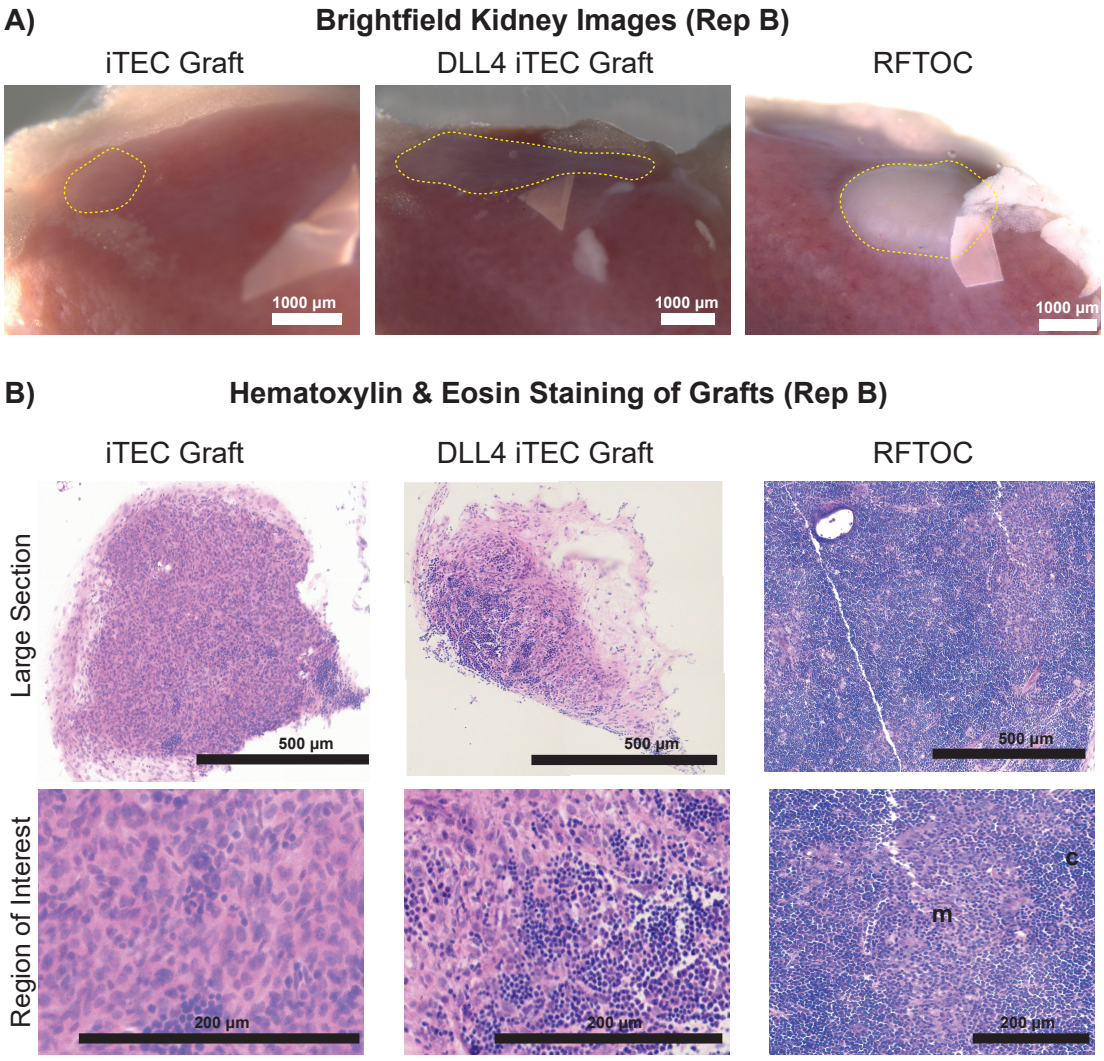


Figure 3.5.9. Histology of recovered grafts two weeks in vivo Rep B. A) Brightfield images of kidneys used for iTEC RTOC, DLL4 iTEC RTOC and RFTOC transplantation in Rep B. Grafts are outlined in yellow lines. **B)** Haematoxylin and eosin (H&E) staining of sectioned recovered grafts. In the top panel the whole area of the isolated sectioned graft is depicted. In the bottom, regions of interest were focused on to show 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. Distinct cortical and medullar areas were not obvious in iTEC or DLL4 iTEC RTOC in Rep B. Data for Cre MEFs were not shown as grafts were too small to isolate from under the kidney capsule and undergo histological processing in the same manner as above.

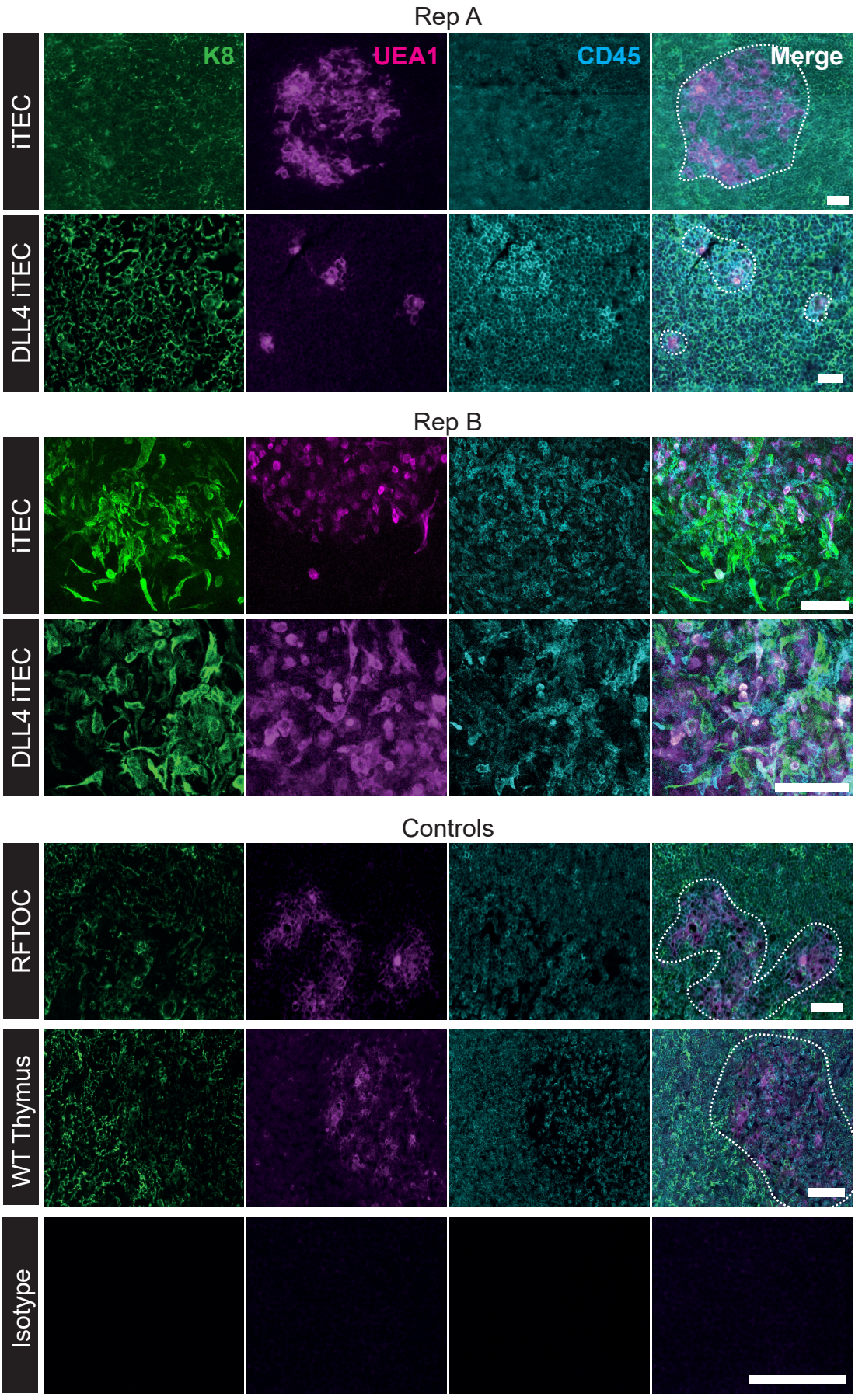

Rep A
K8
UEA1
CD45
Merge
iTEC
DLL4 iTEC
Rep B
iTEC
DLL4 iTEC
Controls
RFTOC
WT Thymus
Isotype

Figure 3.5.10. Immunohistochemistry staining of recovered grafts two 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 ROIs were selected to showcase whether successful organisation and distinct cortical and medullary regions had formed in the graft. Positive controls include recovered RFTOC and a 4 week female WT thymus. The negative control used was stained for isotypes of the primary antibody, where possible, and followed by secondary antibody staining. iTEC indicates where the host received an iTEC RTOC and DLL4 iTEC indicates where the host received a DLL4 iTEC RTOC. Images for both Rep A and Rep B of iTEC and DLL4 conditions are included to show variation between biological replicates on outcome of histology. Scale bar 100 μm.

3.6. Discussion

In this chapter, I investigated the effects of changing three parameters in the iTEC reprogramming protocol on the functionality of the resulting iTEC: (i) prolonging the time in which MEFs undergoing reprogramming were exposed to FOXN1 before harvesting, (ii) increasing the reaggregation period in RTOC prior to transplantation, and (iii) enriching for DLL4-positive cells among iTEC.

3.6.A. Effect of changing reprogramming time

The data presented above show that MEFs exposed to FOXN1 for 18 days show morphology more suggestive of epithelial identity than those recovered after 4 days, and also resulted in an increased likelihood of generating grafts that could be recovered 2 weeks after transplantation. Based on this, the 18 day reprogramming protocol was taken forward for all subsequent experiments. It will be of interest in future experiments to fully elucidate the differences between these early iTEC and late iTEC, for instance with single cell RNA-sequencing experiments. Establishing the differences in their gene expression profile may lead to a better understanding of the molecular mechanisms underpinning FOXN1-mediated reprogramming.

It is of interest that the frequency of GFP^+ cells, representing iTEC, is improved after eighteen days in comparison to the initial timepoint of four days. The underlying mechanism for this is not known, however, proliferation of GFP^+ cells, increased time of exposure to FOXN1, or increasing FOXN1 levels over time may be responsible. As the mechanism of conversion of MEFs to iTEC is likely a multistep process of direct Foxn1 and indirect Foxn1 target activation, we favour the hypothesis that a longer period of time is required for activation of pathways downstream of FOXN1 that are required for successful iTEC differentiation.

It is important to note that although GFP is utilised to report FOXN1 levels present in the reprogramming cells, and the two genes are transcribed in a bi-cistronic message separated by an IRES element, the levels of GFP and FOXN1 may not directly correspond. For instance, the two proteins are likely to have different half-lives. GFP has a stable half-life of approximately 26 hours and therefore a lag in GFP degradation in comparison to FOXN1 (whose exact half-life has yet to be determined) may be possible (Corish and Tyler-Smith, 1999). This might explain the large variation in *Foxn1* mRNA levels apparent in Figure 3.5.2.; alternatively, it is possible that the outlier data represent a technical error. The latter could be determined by further repetitions of this experiment.

3.6.B. Effect of changing period of reaggregation in culture prior to transplantation

Bredenkamp *et al*., (2014) and Sheridan *et al*., (2009) reported that a 12-16 hour reaggregation period for RTOC was sufficient before transplantation, and this was initially employed in these experiments. However, I noted that upon transfer from cell culture to the kidney capsule, manual manipulation often resulted in destruction of RTOC structures and therefore tested the outcome of increasing the reaggregation time to 60 hours. This resulted in RTOC that were easier to handle for transplantation, and subsequent improved recovery grafts from under the kidney capsule.

The RTOC method relies on purifying populations by flow cytometry from primary tissue or *in vitro* cultures that have undergone enzymatic digestion. Thus, reaggregation of the different input cell populations will rely on cell surface adhesion molecules and, probably later, on ECM. The compaction reaggregation method used herein was originally developed to minimise reaggregation time and has successfully been used to allow reaggregate cultures to be generated from previously refractory tissues (Sheridan *et al*., 2009) as well as thymic tissue. We assume that the requirement for a longer reaggregation period in our current experiments than that we originally reported reflects changes in the adhesion profile of the iTEC, resulting from genetic changes in house C57BL/6J line or from changes in the enzyme digestion protocol, and/or from changes in ECM production within the reaggregates.

The cellular structures first formed following compaction reaggregation do not have surface adhesion molecules and ECM present. The time required for ECM to form varies based on the cell type producing it and is influenced by mechanical stimuli (Franco-Barraza *et al*., 2016). One group studying 3-D ECM matrices obtained from fibroblasts reported that 5-9 days of culture prior to collecting ECM was appropriate for obtaining a sufficiently thick matrix (Hozumi *et al*., 2008; Koch *et al*., 2008). The group does note however that the time required for each type of fibroblast to generate ECM varies and time course experiments should be carried out. If 5-9 days is required for a thick matrix that can be manipulated, then integrity of structures will most likely increase if the 12-16 h is also increased.

Analysis of the adhesion molecule profile of iTEC and of ECM deposition in RTOC would be of interest but was outside the scope of this thesis.

3.6.C. Effect of enriching for DLL4$^+$ cells among iTEC

Enriching for GFP$^+$DLL4$^+$ iTEC prior to transplantation did not improve the phenotype or functionality of the grafts versus GFP$^+$ iFoxn1 MEFs, in terms of capacity to support thymopoiesis or to generate grafts with clear cortex and medullary compartments.

As DLL4 is a downstream target of FOXN1 it was thought that selecting a population that was further refined and had activated the FOXN1 transcriptional program may be more efficient and reduce variability within the system. DLL4 expressed on TEC interacts with Notch1 on thymocytes, activates Notch signalling and is indispensable for T cell lineage commitment (Hirano *et al.*, 2015). Notch signalling is required iteratively throughout thymopoiesis. For instance, its downstream targets such as c-Myc have been found to be important in the DN3 to DP progression (Hozumi *et al*., 2008; Ribeiro *et al*., 2013). Therefore, the lack of overt effect of selection for DLL4$^+$ cells was surprising.

Investigation of the gene expression profiles of DLL4$^+$ versus GFP$^+$ whole iTEC did not reveal clear differences, due to variability in gene expression levels between experiments. This likely reflected the fact that many of the cells in the GFP$^+$ iTEC expressed DLL4 (Figure 3.5.1). However, as a trend, DLL4$^+$ iTEC appeared to show increased expression of *Dll4, Dll1, Flt3-L, Kit-L, Ccl25, Psmb11,* suggesting they expressed higher levels of cTEC-associated genes. *Dll4, Flt3l* and *Kitl* have been shown to be important in early stages of thymopoiesis, with *Dll4* and *Kitl* acting at the DN1/DN2 phase (Rodewald *et al*., 1995; Koch *et al*., 2008; Buono *et al*., 2016), and *Flt3l* acting on the earliest thymic lymphoid progenitors (Sitnicka *et al*., 2007). The lack off effect of DLL4$^+$ iTEC on thymopoiesis may be attributed to the fact that RTOC generated contained total DN thymocytes rather than earlier precursors. Additionally, both iTEC and DLL4$^+$ iTEC expressed *Dll4, Dll1, Flt3l, Kitl, Ccl25* and *Psmb11* at much higher levels than TEPC, so these are unlikely to be limiting in the test populations.

Analysis of thymocyte development in iTEC versus DLL4 iTEC showed no difference in the DN to DP stages. Interestingly though, DLL4 iTEC (9.17%, 81.32%, 94.38% DP) did appear to show larger variability than the iTEC condition (92.16%,

93.54%, 86.64% DP) in percentage of DP cells present. Although DLL4 should show improved T cell lineage commitment these results can be explained by the fact that GFP^+ iTEC also contain $DLL4^+$ iTEC within them so they are not lacking it from the system and additionally there is the presence of already committed T cells present in our system.

It was of concern that in enriching for DLL4, we might prejudice the production of cTEC over mTEC in the resulting grafts. High DLL4 expression is specifically associated with cTEC, whereas low DLL4 expression is noted in mTEC (Koch *et al*., 2008). Interestingly, histology results revealed that DLL4 iTEC were able to give rise to both cTEC and mTEC with self-organisation taking place. The latter could be explained by varying levels of DLL4 expression on iTEC found in the DLL4 gate used during FACS, with high levels of DLL4 associated with cTEC and low levels with occurring mTEC. It would be of interest to analyse additional grafts to determine whether medullary areas are larger in grafts generated from iTEC compared to $DLL4^+$ iTEC, as suggested by this experiment, albeit with N of one for each condition.

3.6.D. Considerations regarding experimental design

Key limitations regarding the system remain and lie within the variability of the phenotype and function of iTEC generated for each experiment. Although the reasons behind this variation remain unclear, they may include the heterogeneous nature of the MEF preparations used to generate iTEC, since batch to batch variation among MEFs has not been well characterised. It is possible that there may be subpopulations of MEFs that are more able to reprogramme into iTEC, and that the relative frequency of these may vary between batches. It is also possible that reprogramming into fully functional iTEC is a relatively rare event (similar to the frequency of reprogramming into truly pluripotent iPS cells, when that protocol was first reported), and that the scarcity of such a fully reprogrammed iTEC population may explain the variation observed. These possibilities will be explored in future experiments. The use of an immortalised fibroblast cell line such as NIH/3T3 was not considered as starting material for these experiments, since previous experiments indicated that NIH/3T3 cells express some FOXN1 target genes, and that these are switched off in NIH/3T3 cells upon enforced expression of FOXN1 (Blackburn lab, unpublished). Furthermore, while the original RFTOC paper included NIH/3T3 cells as the mesenchymal cell component (Anderson *et al*., 1993), we note

that the RFTOC were not grafted in that publication. Previous experiments carried out by this lab found that RFTOC that included non-irradiated NIH/3T3 MEFs gave rise to tumours upon transplantation, and this provided the rationale for using MEFs rather than NIH/3T3 in RFTOC in our original publication of this system (Bennett *et al*, 2002). The tumorigenicity of NIH/3T3 has been reported elsewhere (Greig *et al*., 1985).

Heterogeneity among newly reprogrammed *in vitro* iTEC, combined with the probability that the high level of FOXN1 expression in iTEC prejudices against proliferation, is likely to affect the efficacy of transplantation. To improve on this, it will be important to dissect the early events in iTEC reprogramming, using RNAseq and chromatin analyses. The use of markers of differentiated TEC markers to enrich for functional iTEC is not possible, as it is already established that iTEC do not express these markers *in vitro*; based on this, iTEC prior to transplantation are thought to resemble immature TEC. Conceptually, most systems for generating organoids build the organoids from stem or progenitor populations, and therefore rather than eliminating immature iTEC subpopulations from the input population for iTEC-RTOC it may be more interesting to explore whether modulating FOXN1 levels allows more efficient generation of progenitor or stem iTEC.

In addition to inherent iTEC reprogramming variation, variability among thymocyte and mesenchymal input populations; other factors including vascularisation of grafts and timing of recruitment of lymphoid progenitors; and technical factors including the mechanical manipulation of RTOC during transfer *in vivo* may all contribute to the variability in outcome of experiments. The protocol for generating RTOC is technically demanding and requires large cell numbers to generate a cell pellet suitable for manual manipulation, introducing further technical variation. As the method of generating RTOC relies on cell types obtained from mouse embryonic thymi in addition to iTEC, this limited the number of conditions that could be examined simultaneously during an experiment. FTM are neural-crest derived and give rise to pericytes, smooth muscle cells, capsular and medullary fibroblasts (Foster *et al*., 2008; Nitta *et al*., 2020). The FTM population may therefore be heterogeneous, and small differences in developmental timing, as occurs within litters, may result in differences between FTM populations that may also introduce variation into the system. The aforementioned factors may contribute to both the technical and biological variation and the abundance of these renders it difficult to

determine the contribution of each to the variation observed at this stage of the project.

In order to determine the variability found within the iTEC population it would be beneficial to have carried out single cell RNA sequencing of the reprogrammed cells at both the day four and day eighteen (and additional) timepoints and compared these data to that obtained from fetal TEC at different stages of development. Comparing iTEC subpopulations established from different batches of MEFs, to that of fetal thymus TEC would offer insight into batch-to-batch variation and may identify rare iTEC subpopulations required for successful engraftment and thymopoiesis in grafts. Use of bar-code-based lineage tracing approaches may also help to understand how functional iTEC are generated, and how they participate in iTEC-RTOC.

The issues of variability between experiments discussed above also made statistical analysis difficult, due to the limited number of biological repeats that could be analysed. By carrying out power analysis calculations in R it was determined that N=16 repeats would have been required in order to state that the probability of seeing a difference between the DP thymocyte population of $DLL4^{+}$ iTEC-RTOC and iTEC-RTOC is 0.8 with significance set to $p=0.05$. Due to the limited tissue availability of FTM required for experiments and the large number of animals that would be required for transplantation, these experimental group sizes could not however be increased in the present study.

3.7. Conclusion

From these data I was able to conclude that a longer reprogramming time (18 days) and a longer reaggregation period (60 hours) were both beneficial, allowing harvesting of more iTEC and better RTOC transplantation and recovery of grafted RTOC. Therefore, these two changes were incorporated into an optimised protocol and used for all subsequent experiments. However, upon enriching iTEC for DLL4-expressing cells, no notable differences were observed in the phenotype or functionality of the cells, with both being capable of mediating efficient thymopoiesis and organisation into cortex and medulla upon transplantation. Therefore, this step was not included in the optimised protocol.

I note that, despite these improvements, the data presented in this Chapter still display batch to batch variation as assessed by both flow cytometry and histology read-outs, highlighting the need for further optimisation of the protocol to reduce variability. One such limiting factor might be the requirement for fetal thymic cells for the generation of RTOC, as this may also introduce variability into the system, and will be explored in following chapters.

In summary, the findings of this Chapter in relation to the hypotheses examined are as follows:

(i) Increasing the iFoxn1 MEF reprogramming time from four to eighteen days resulted in an increase in number of functional GFP^+ iTEC with better TEC-like morphology.

(ii) Increasing the reaggregation period of iTEC-RTOC from eighteen to sixty hours resulted in successful recovery of macroscopic grafts.

(iii) Enriching for iTEC that express DLL4 did not appear to enhance TEC-like phenotype or functionality.