6.1.B. Experimental design

iTEC-MultiRTOC, Cre MEF-MultiRTOC or RFTOC were transplanted under the kidney capsule of *Foxn1*$^{G/G}$ mice using the optimised protocol discussed in Chapter 5. Note that these iTEC-RTOC did not contain fetal thymic mesenchyme. Peripheral blood samples were collected bi-weekly from four to sixteen weeks post-transplantation and mice were maintained for a total of twenty-two weeks post-transplantation which constituted the experimental endpoint. Inguinal, cervical and axillary lymph nodes, and spleen, were collected from each recipient and processed for flow cytometric analysis. The grafts were also recovered and processed for histological and immunohistochemical analysis. The transplant conditions and experimental strategy are outlined in Figure 6.2.

Peripheral blood, lymph node and spleen cells were analysed using the following FACS panel: ZombieGreen fixable viability dye, α-CD3ε, α-TCRβ, α-CD4, α-CD8, α-CD44, α-CD62L, α-CD25, α-CD127, α-TCRγδ, Ki67, α-FOXP3. Zombie Green was used to exclude dead cells from the analysis. CD3ε, TCRβ and TCRγδ staining was used to identify αβ T cells (CD3ε$^+$TCRβ$^+$) and γδ T cells (CD3ε$^+$TCRγδ$^+$). Regulatory T cells (Tregs) were gated as CD25$^+$CD4$^+$FOXP3$^+$ αβ T cells. CD25 (receptor for IL-2) and CD127 (receptor for IL-7) were used to distinguish effector T cells (Teff: CD25$^-$CD127$^-$) from naïve and central memory T cells (Tn & Tcm: CD25$^-$ CD127$^+$) (Liu et al., 2006; Seddiki et al., 2006; Hartigan-O'Connor et al., 2007; Dunham et al., 2008). CD44, an antigen experienced T cell marker (DeGrendele, Estess and Siegelman, 1997; Goldrath, Bogatzki and Bevan, 2000) and CD62L, a naïve T cell marker (Yang et al., 2011) were used to distinguish naïve (CD44$^-$CD62L $^+$) from antigen experienced (CD44$^+$CD62L$^-$) T cells (Gerberick et al., 1997). Ki67 was included to allow identification of proliferating cells. Gates were set based on FMO controls (Figure 6.3).

Analysis of WT or non-transplanted *Foxn1*$^{G/G}$ mice lymph nodes and spleen is shown in Figures 6.4 – 6.7. The absolute numbers of each T cell subset present in the mice can be seen in Tables 6.1 (lymph nodes) and 6.2 (spleen). For the transplantation experiments, a total of three fully independent experiments (i.e grafting on different days with different iTEC preparations) were carried out. Due to variability, each mouse was examined as a biological replicate even if it was transplanted on the same day as another mouse with the same batch of iTEC. A

total of five mice received the Cre MEF or RFTOC condition and seven mice received iTEC.

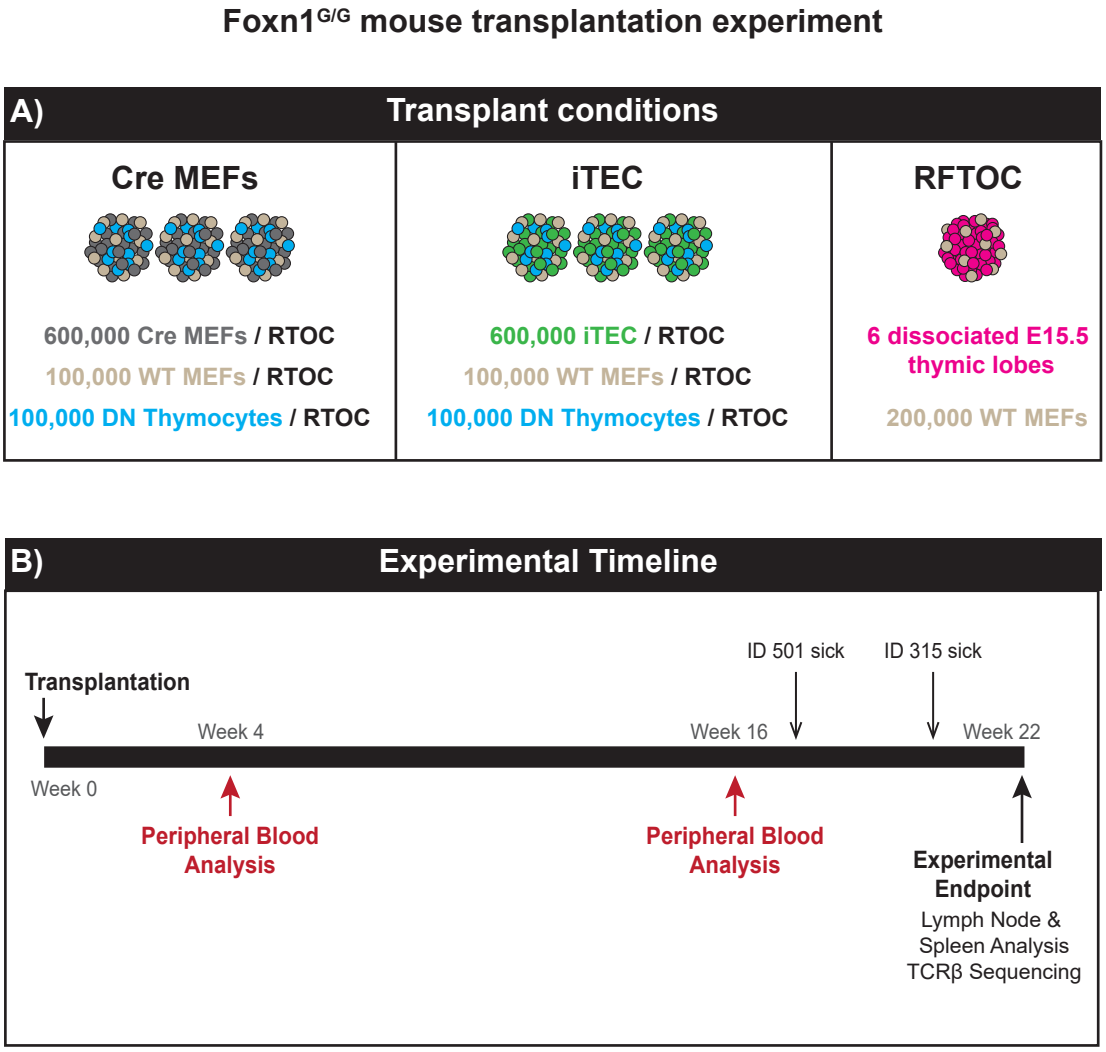


Figure 6.2. Experimental design for *Foxn1*$^{G/G}$ mouse transplantation. A) RTOC conditions transplanted into nude *Foxn1*$^{G/G}$ mice. Detailed description of cells and numbers transplanted in each condition. **B)** Experimental timeline: Mice were transplanted with RTOC as stated above and peripheral blood T cell analysis was carried out at 4 and 16 weeks post-transplantation. Mice with IDs 501 and 315 were culled at 17 and 20 weeks, respectively due to rapid weight loss. At 22 weeks all mice were culled and lymph node and spleen T cell analysis was carried out. Lymph node and spleen T cells were collected for TCR Vβ amplicon generation for RNA-sequencing to determine T cell repertoire diversity of animals.

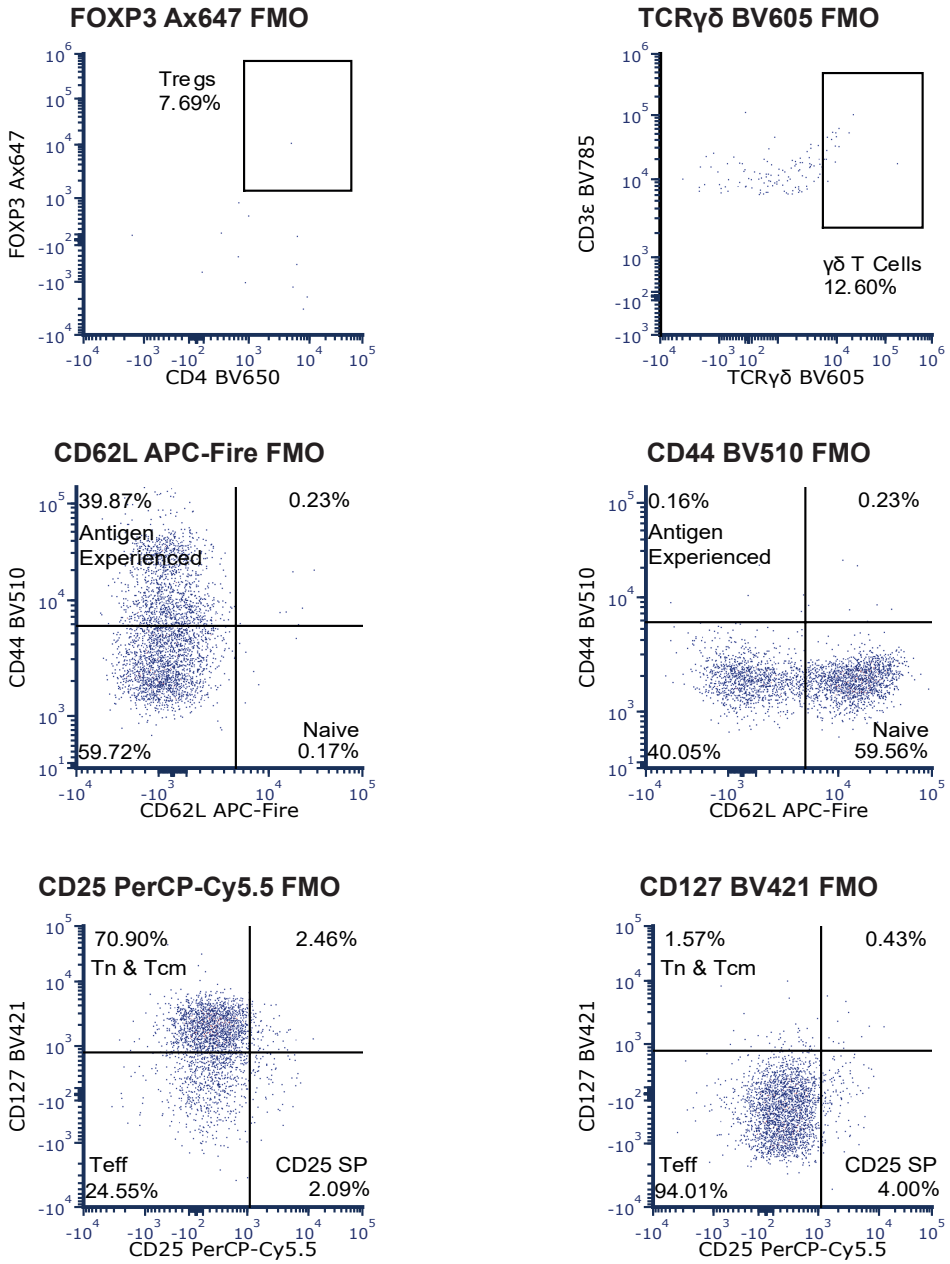
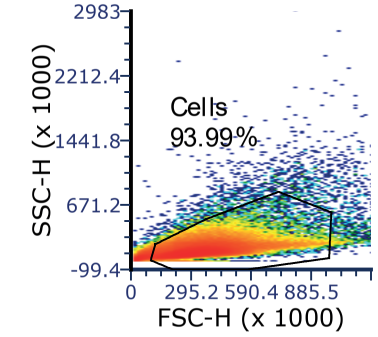
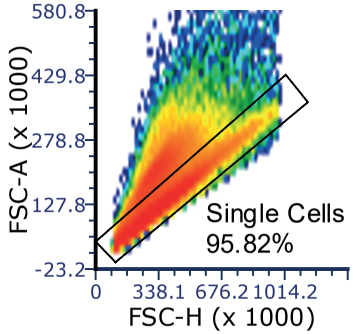
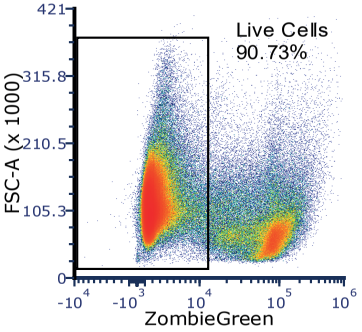
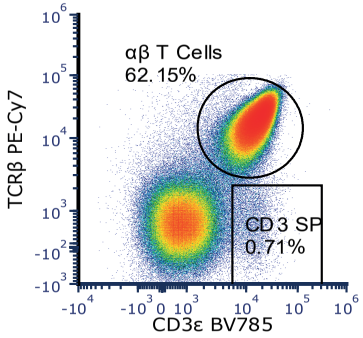


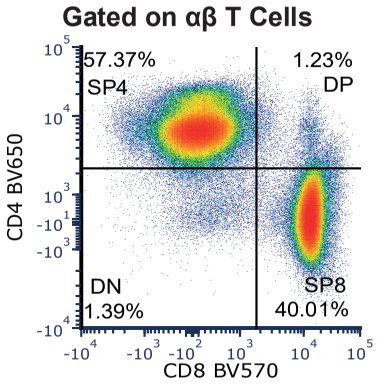
Figure 6.3. Fluorescence Minus One (FMO) controls for staining panel used to analyse *Foxn1*$^{G/G}$ peripheral T cells. Representative FACS plots for FOXP3 Ax647, TCRγδ BV605, CD62L APC-Fire, CD44 BV510, CD25 PerCP-Cy5.5, CD127 BV421 antibody FMOs.

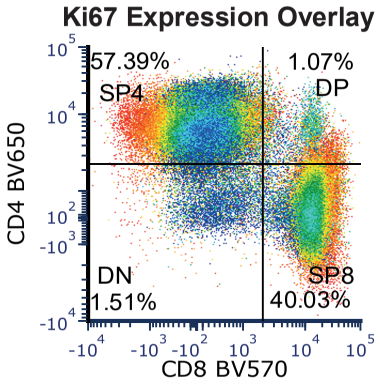

SSC-H (x 1000)
2983
2212.4
1441.8
671.2
-99.4
Cells
93.99%
0
295.2 590.4 885.5
FSC-H (x 1000)

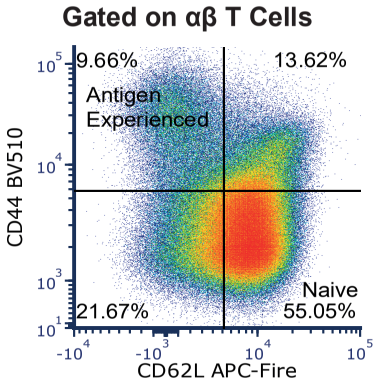

FSC-A (x 1000)
580.8
429.8
278.8
127.8
-23.2
Single Cells
95.82%
0
338.1 676.2 1014.2
FSC-H (x 1000)

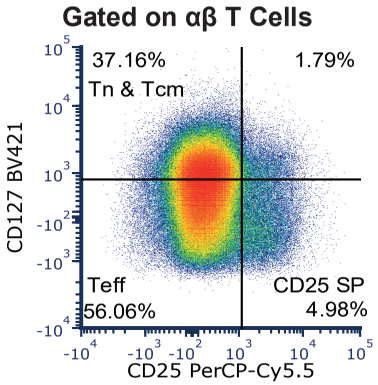

FSC-A (x 1000)
421
315.8
210.5
105.3
Live Cells
90.73%
ZombieGreen

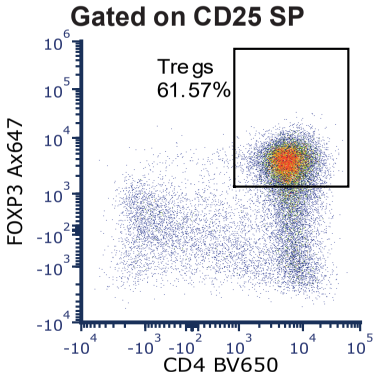

TCRβ PE-Cy7
αβ T Cells
62.15%
CD3 SP
0.71%
CD3ε BV785

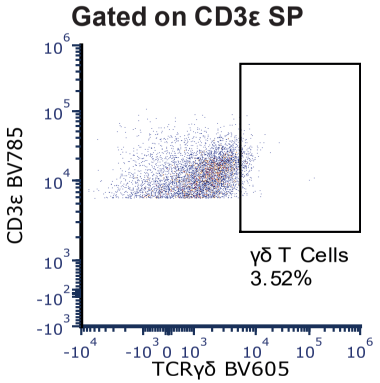

Gated on αβ T Cells
CD4 BV650
57.37%
SP4
1.23%
DP
DN
1.39%
SP8
40.01%
CD8 BV570

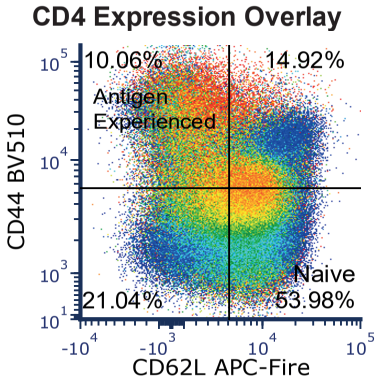

Ki67 Expression Overlay
CD4 BV650
57.39%
SP4
1.07%
DP
DN
1.51%
SP8
40.03%
CD8 BV570

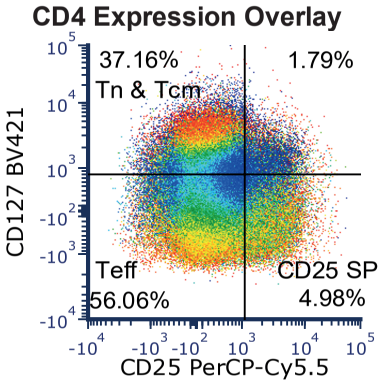

Gated on αβ T Cells
CD44 BV510
9.66%
13.62%
Antigen
Experienced
21.67%
Naive
55.05%
CD62L APC-Fire


Gated on αβ T Cells
CD127 BV421
37.16%
Tn & Tcm
1.79%
Teff
56.06%
CD25 SP
4.98%
CD25 PerCP-Cy5.5


Gated on CD25 SP
FOXP3 Ax647
Tregs
61.57%
CD4 BV650


Gated on CD3ε SP
CD3ε BV785
γδ T Cells
3.52%
TCRγδ BV605


CD4 Expression Overlay
CD44 BV510
10.06%
14.92%
Antigen
Experienced
21.04%
Naive
53.98%
CD62L APC-Fire


CD4 Expression Overlay
CD127 BV421
37.16%
Tn & Tcm
1.79%
Teff
56.06%
CD25 SP
4.98%
CD25 PerCP-Cy5.5

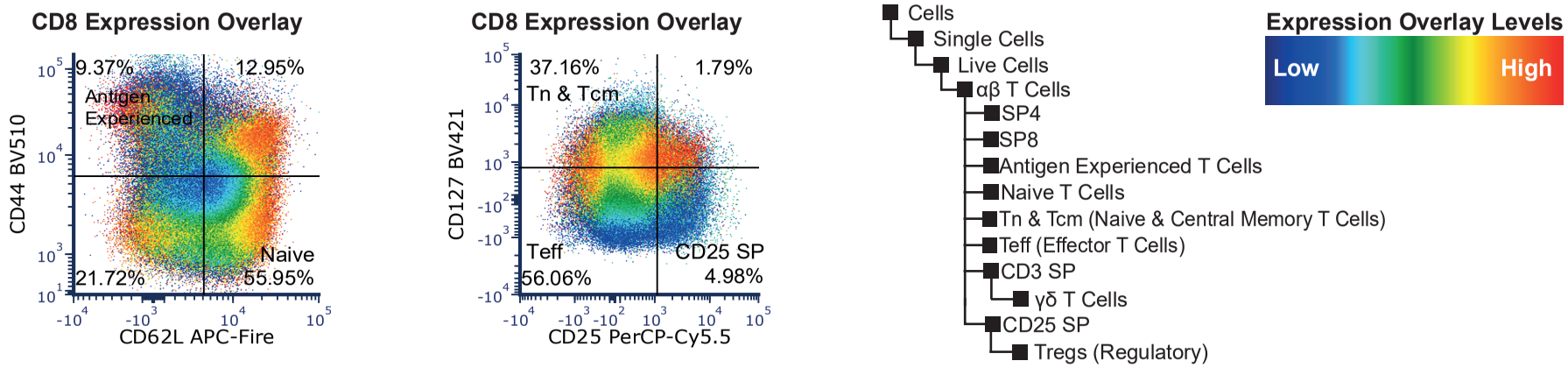
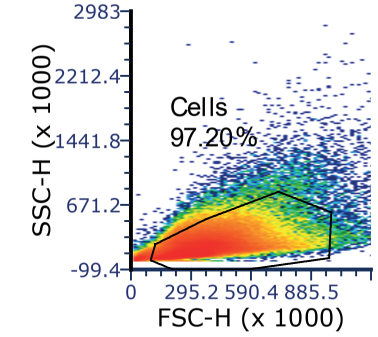
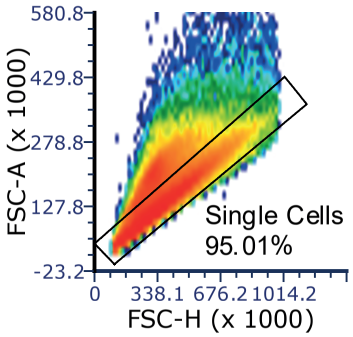
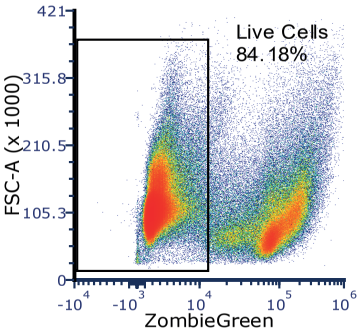
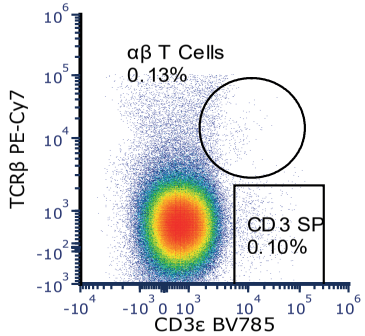


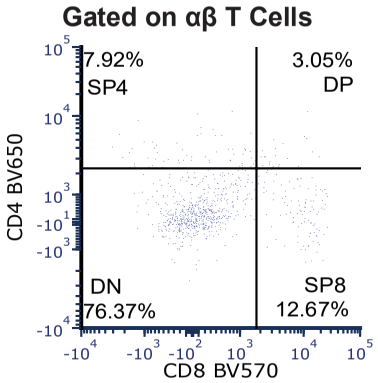
Figure 6.4. Validation of staining panel used to analyse *Foxn1*$^{G/G}$ mice T cells: WT lymph node. Lymph node T cell analysis of male 6 week old WT C57BL/6 mouse. Axillary, inguinal and cervical lymph nodes were collected.

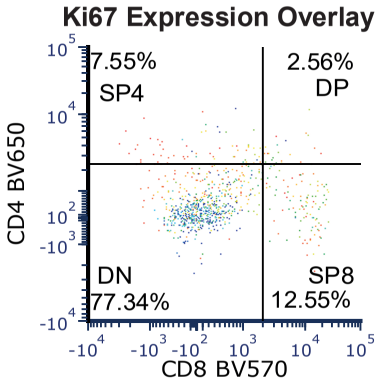

Cells
97.20%
SSC-H (x 1000)
FSC-H (x 1000)

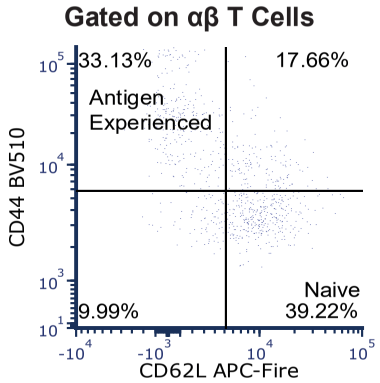

Single Cells
95.01%
FSC-A (x 1000)
FSC-H (x 1000)

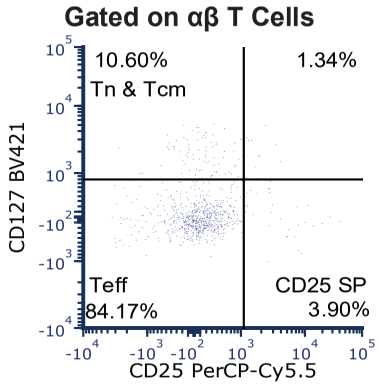

Live Cells
84.18%
FSC-A (x 1000)
ZombieGreen

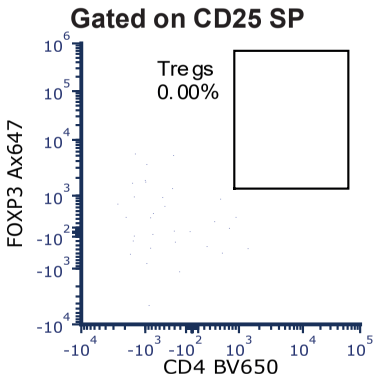

αβ T Cells
0.13%
CD3 SP
0.10%
TCRβ PE-Cy7
CD3ε BV785

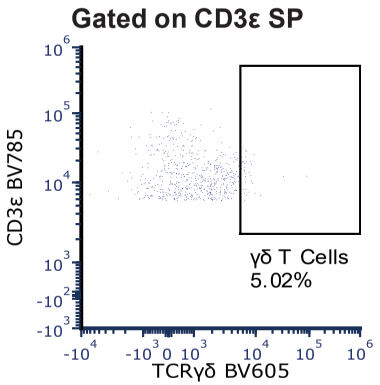

Gated on αβ T Cells
7.92%
SP4
3.05%
DP
DN
76.37%
SP8
12.67%
CD4 BV650
CD8 BV570

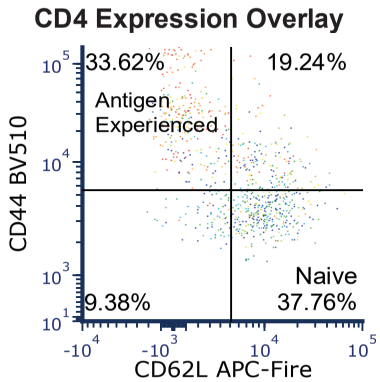

Ki67 Expression Overlay
7.55%
SP4
2.56%
DP
DN
77.34%
SP8
12.55%
CD4 BV650
CD8 BV570

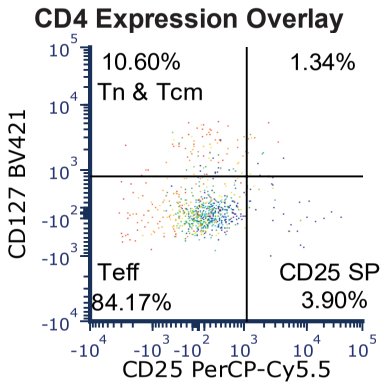

Gated on αβ T Cells
33.13%
17.66%
Antigen
Experienced
9.99%
Naive
39.22%
CD44 BV510
CD62L APC-Fire


Gated on αβ T Cells
10.60%
1.34%
Tn & Tcm
Teff
84.17%
CD25 SP
3.90%
CD127 BV421
CD25 PerCP-Cy5.5


Gated on CD25 SP
Tregs
0.00%
FOXP3 Ax647
CD4 BV650


Gated on CD3ε SP
γδ T Cells
5.02%
CD3ε BV785
TCRγδ BV605


CD4 Expression Overlay
33.62%
19.24%
Antigen
Experienced
9.38%
Naive
37.76%
CD44 BV510
CD62L APC-Fire


CD4 Expression Overlay
10.60%
1.34%
Tn & Tcm
Teff
84.17%
CD25 SP
3.90%
CD127 BV421
CD25 PerCP-Cy5.5

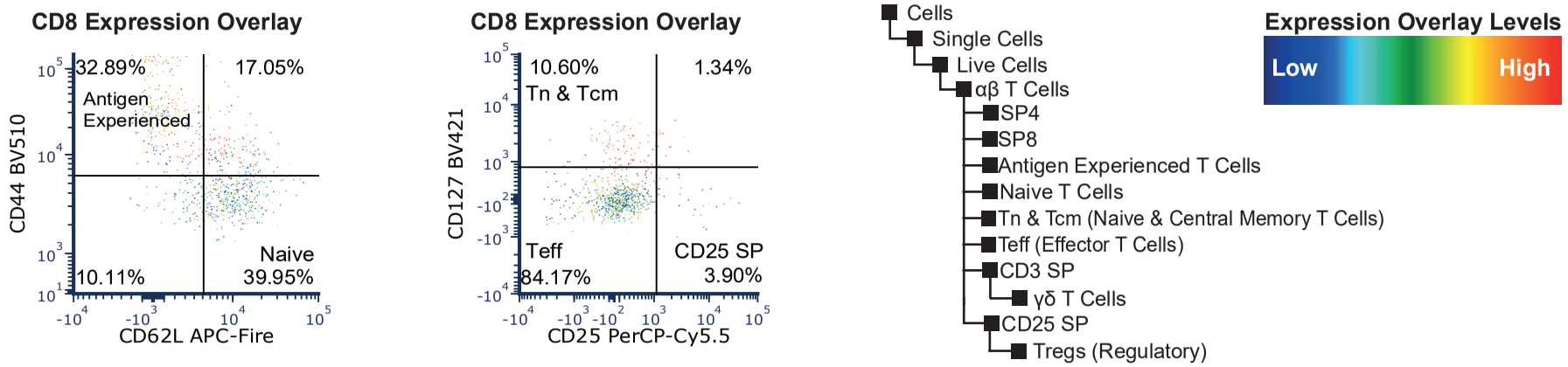
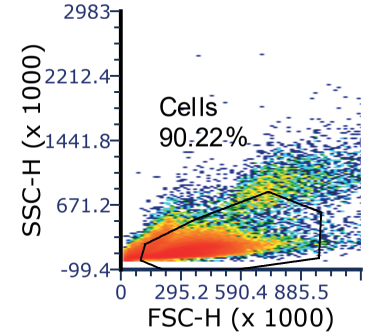
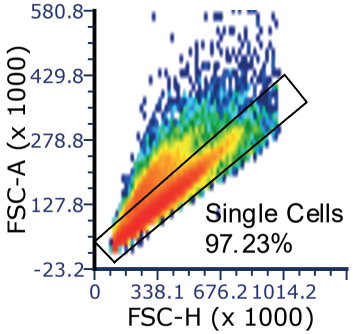
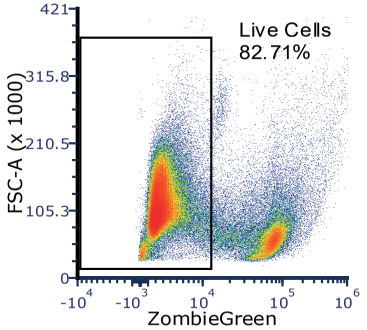
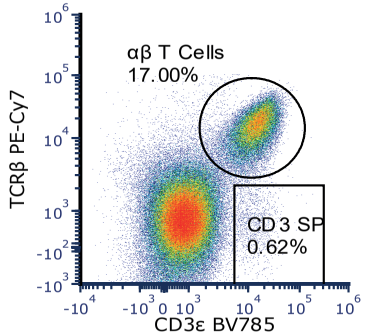


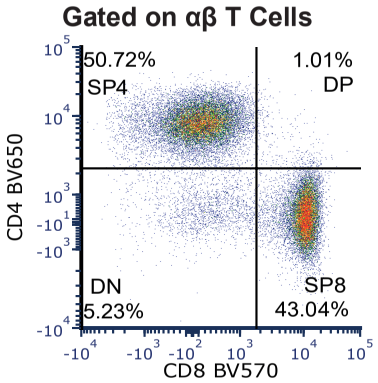
Figure 6.5. Non-transplanted *Foxn1*$^{G/G}$ lymph node T cell analysis. FACS plots and gating hierarchy for lymph node T cells of non-transplanted *Foxn1*$^{G/G}$ male 13 week old mouse. Axillary, inguinal and cervical lymph nodes were collected.

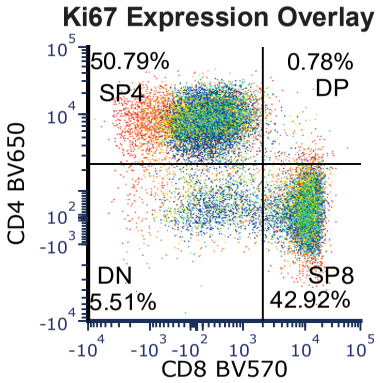

SSC-H (x 1000)
2983
2212.4
1441.8
671.2
-99.4
Cells
90.22%
0
295.2 590.4 885.5
FSC-H (x 1000)

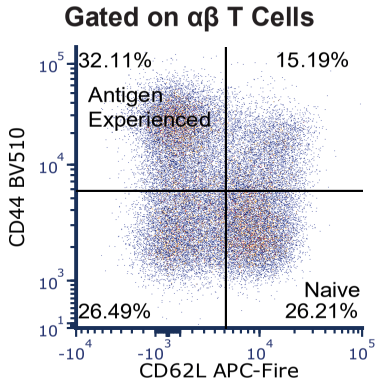

FSC-A (x 1000)
580.8
429.8
278.8
127.8
-23.2
Single Cells
97.23%
0
338.1 676.2 1014.2
FSC-H (x 1000)

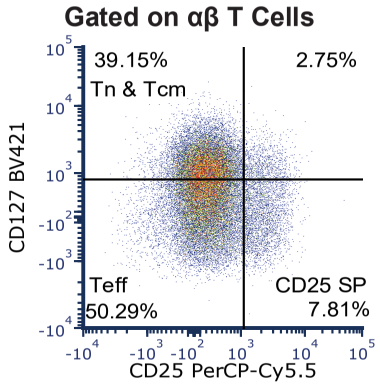

FSC-A (x 1000)
421
315.8
210.5
105.3
0
Live Cells
82.71%
ZombieGreen

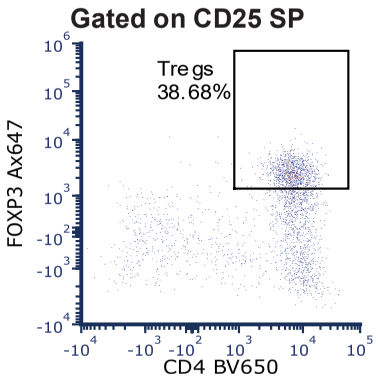

TCRβ PE-Cy7
αβ T Cells
17.00%
CD3 SP
0.62%
CD3ε BV785

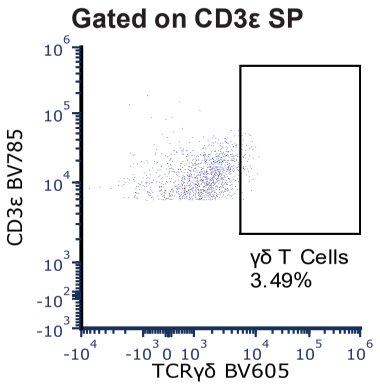

Gated on αβ T Cells
50.72%
SP4
1.01%
DP
DN
5.23%
SP8
43.04%
CD4 BV650
CD8 BV570

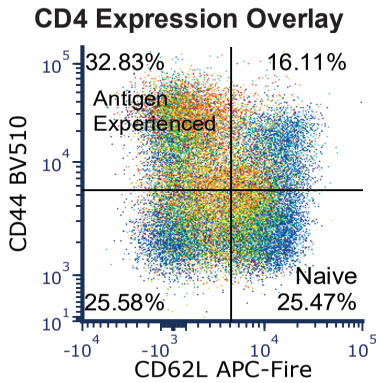

Ki67 Expression Overlay
50.79%
SP4
0.78%
DP
DN
5.51%
SP8
42.92%
CD4 BV650
CD8 BV570

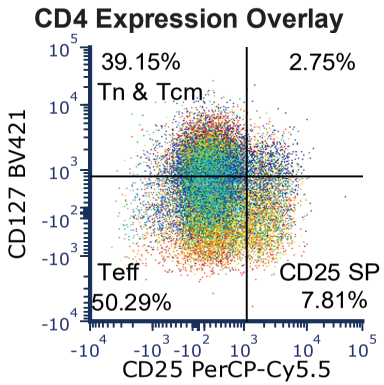

Gated on αβ T Cells
32.11%
15.19%
Antigen
Experienced
26.49%
Naive
26.21%
CD44 BV510
CD62L APC-Fire


Gated on αβ T Cells
39.15%
2.75%
Tn & Tcm
Teff
50.29%
CD25 SP
7.81%
CD127 BV421
CD25 PerCP-Cy5.5


Gated on CD25 SP
Tregs
38.68%
FOXP3 Ax647
CD4 BV650


Gated on CD3ε SP
γδ T Cells
3.49%
CD3ε BV785
TCRγδ BV605


CD4 Expression Overlay
32.83%
16.11%
Antigen
Experienced
25.58%
Naive
25.47%
CD44 BV510
CD62L APC-Fire


CD4 Expression Overlay
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Tn & Tcm
Teff
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CD25 SP
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CD127 BV421
CD25 PerCP-Cy5.5

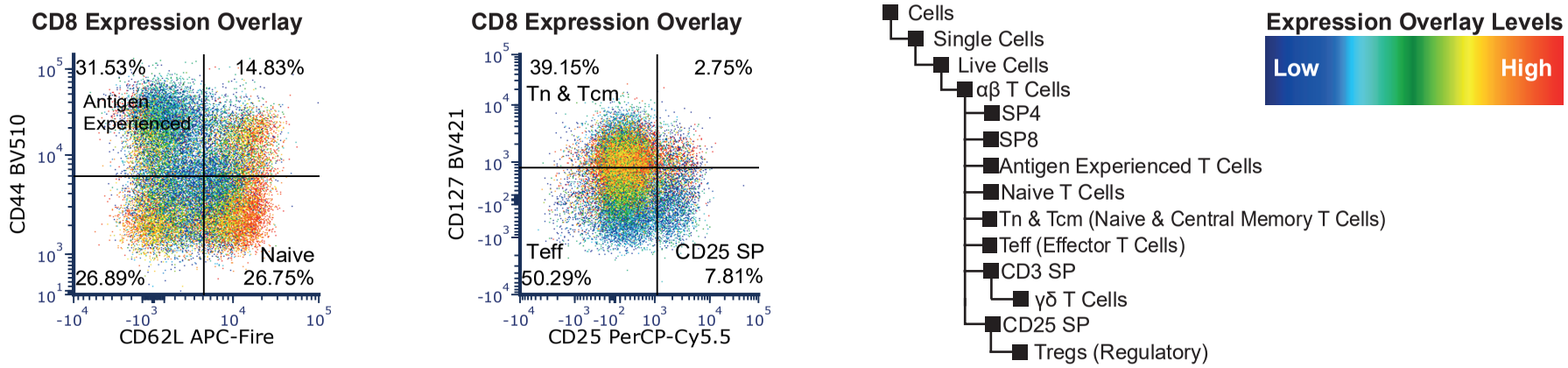
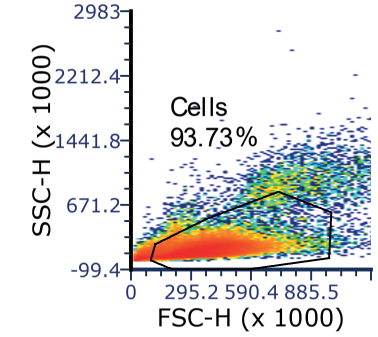


Figure 6.6. Validation of staining panel used to analyse *Foxn1*$^{G/G}$ mice T cells: WT spleen. Spleen T cell analysis of 6 week old male C57BL/6 mouse.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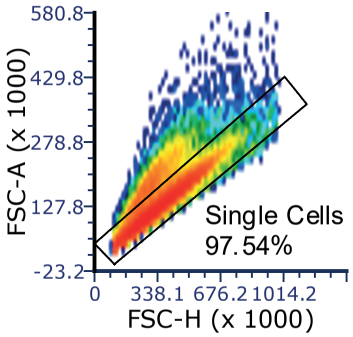
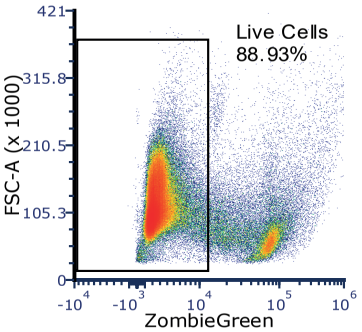
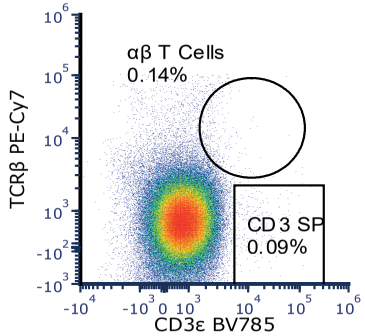
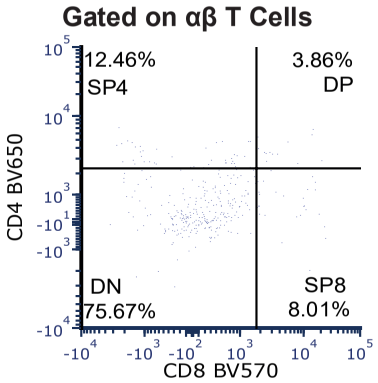
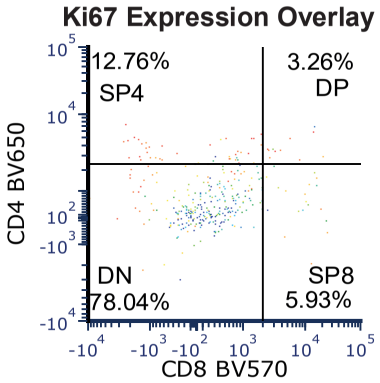
SSC-H (x 1000)
FSC-H (x 1000)


Single Cells
97.54%
FSC-A (x 1000)
FSC-H (x 1000)


Live Cells
88.93%
FSC-A (x 1000)
ZombieGreen


αβ T Cells
0.14%
CD3 SP
0.09%
TCRβ PE-Cy7
CD3ε BV785


Gated on αβ T Cells
12.46%
SP4
3.86%
DP
DN
75.67%
SP8
8.01%
CD4 BV650
CD8 BV570


Ki67 Expression Overlay
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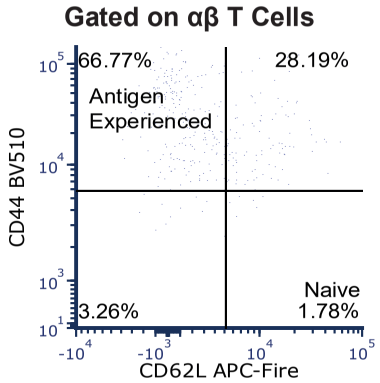
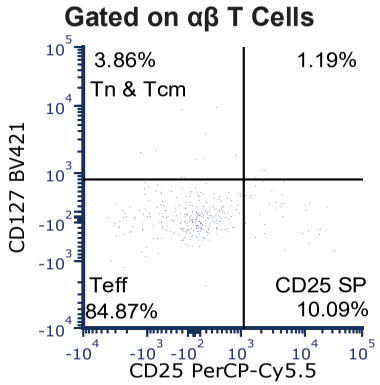
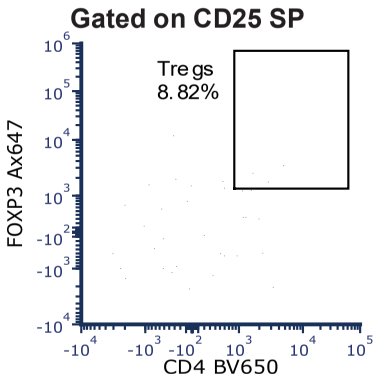
SP4
3.26%
DP
DN
78.04%
SP8
5.93%
CD4 BV650
CD8 BV570


Gated on αβ T Cells
66.77%
28.19%
Antigen
Experienced
3.26%
Naive
1.78%
CD44 BV510
CD62L APC-Fire


Gated on αβ T Cells
3.86%
1.19%
Tn & Tcm
Teff
84.87%
CD25 SP
10.09%
CD127 BV421
CD25 PerCP-Cy5.5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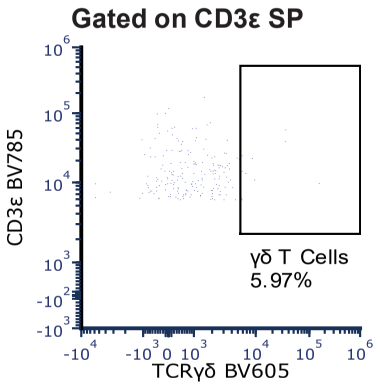
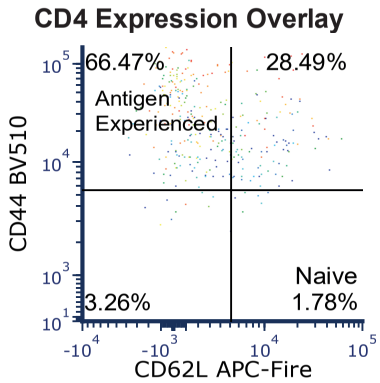
Tregs
8.82%
FOXP3 Ax647
CD4 BV650


Gated on CD3ε SP
γδ T Cells
5.97%
CD3ε BV785
TCRγδ BV605


CD4 Expression Overlay
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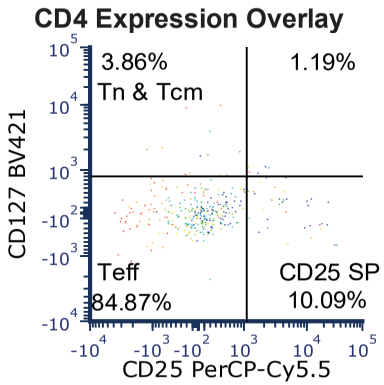
28.49%
Antigen
Experienced
3.26%
Naive
1.78%
CD44 BV510
CD62L APC-Fire


CD4 Expression Overlay
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1.19%
Tn & Tcm
Teff
84.87%
CD25 SP
10.09%
CD127 BV421
CD25 PerCP-Cy5.5

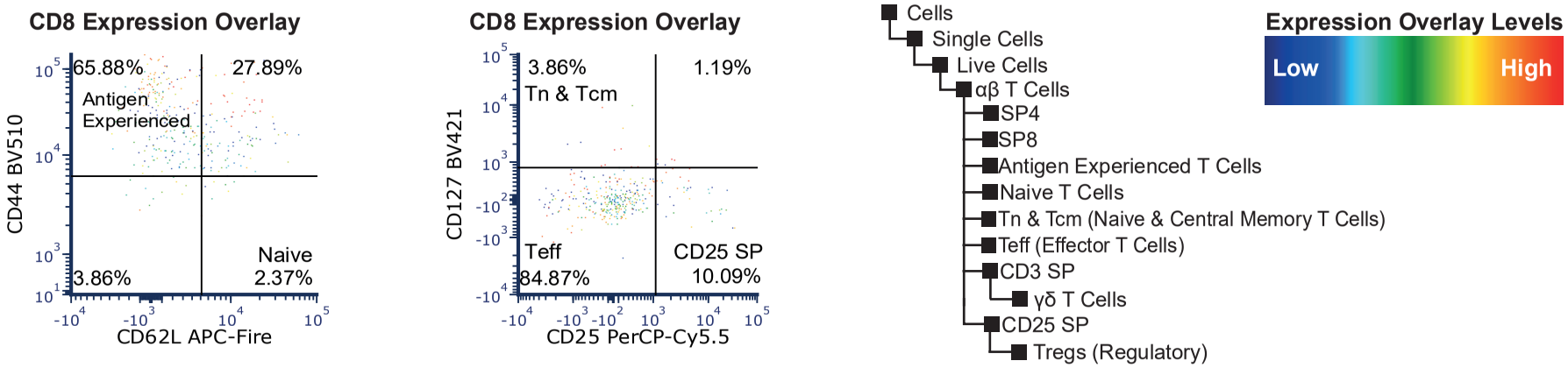


Figure 6.7. Non-transplanted *Foxn1*$^{G/G}$ spleen T cell analysis. FACS plots and gating hierarchy for spleen T cells of non-transplanted *Foxn1*$^{G/G}$ male 13 week old mouse.

Strain	Animal	Age	αβ T cells								CD3 SP
			Total	SP4	SP8	Regulatory	Naïve	Naïve & central memory	Effector	Antigen Experienced	γδ T cells
Foxn1G/G	1	10 wk	1437	114	182	0	564	152	1,209	476	56
	2	13 wk	2200	130	714	4	557	294	1,705	651	54
		Mean	**1818**	**122**	**448**	**2**	**560**	**223**	**1,457**	**564**	**55**
WT	1	6 wk	1,725,514	989,965	690,337	52,934	949,820	641,197	967,404	166,768	693
	2	6 wk	1,417,589	801,423	570,948	38,791	610,512	441,700	897,724	113,363	200
		Mean	**1,571,552**	**895,694**	**630,642**	**45,862**	**780,166**	**541,448**	**932,564**	**140,066**	**446**

Table 6.1. Absolute numbers of lymph node T cells in non-transplanted WT and *Foxn1*$^{G/G}$ mice.

Strain	Animal	Age	αβ T cells								CD3 SP
			Total	SP4	SP8	Regulatory	Naïve	Naïve & central memory	Effector	Antigen Experienced	γδ T cells
Foxn1G/G	1	10 wk	3,370	420	270	30	60	130	2,860	2,250	120
	2	13 wk	8,560	650	1,680	180	360	920	6,740	5,470	340
		Mean	**5,965**	**535**	**975**	**105**	**210**	**525**	**4,800**	**3,860**	**230**
WT	1	6 wk	345,490	175,230	148,700	10,440	90,550	135,250	173,760	110,940	440
	2	6 wk	293,460	152,070	113,980	7,310	74,320	114,920	152,830	99,370	360
		Mean	**319,475**	**163,650**	**131,340**	**8,875**	**82,435**	**125,085**	**163,295**	**105,155**	**400**

Table 6.2. Absolute numbers of splenic T cells in non-transplanted WT vs *Foxn1*$^{G/G}$ mice.

6.2. Analysis of peripheral T cell populations in iTEC-RTOC recipient mice

6.2.A. Peripheral blood analysis

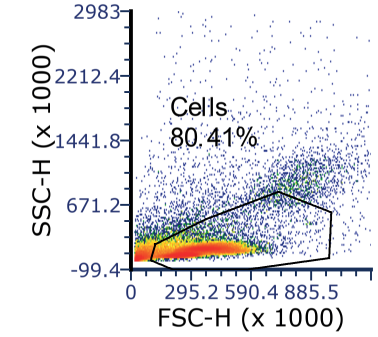
Figures 6.8 – 6.13 show the gating strategy used for peripheral blood T cell analysis, and representative FACS plots from mice analysed at four- and sixteen-weeks post-transplantation, for positive control RFTOC, negative control Cre MEF-MultiRTOC and iTEC-MultiRTOC recipients. Absolute cell numbers were calculated and adjusted to represent numbers per one ml of peripheral blood. For each condition, the absolute cell numbers of specific T cell populations (αβ, SP4, SP8, regulatory, γδ, naïve, naïve and central memory, effector and antigen experienced T cells) present in the peripheral blood at four and sixteen weeks are shown in Table 6.3 and 6.4, and in Figure 6.14.

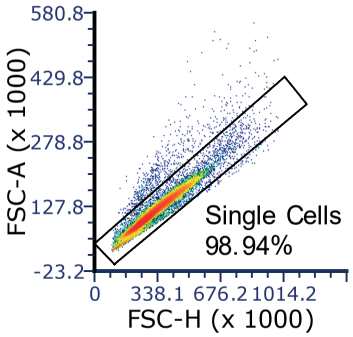
Overall, mice that received RFTOC grafts displayed higher mean numbers of all T cells subsets than Cre MEF or iTEC-RTOC recipients, at both timepoints analysed. The mean number of αβ, SP8, γδ, naïve, naïve and central memory T cell subsets increased over time in RFTOC recipients. iTEC-RTOC recipients had higher numbers of αβ, SP4, SP8, regulatory, naïve and central memory, effector and antigen experienced T cells than Cre MEF recipients at sixteen weeks, but not at four weeks, post grafting. iTEC recipients also showed an increase in all T cell subsets except naïve T cells present in peripheral blood at sixteen weeks compared to four weeks post grafting. Slight increases in cell numbers over time were also observed in Cre MEFs for αβ, SP8 and naïve and central memory T cells, most likely due to the development of extrathymic T cells and homeostatic expansion. Extrathymic T cells found in nude athymic mice are thought to develop in the gut and appear to mainly consist of SP8 T cells (Kennedy, Pierce and Lake, 1992), in line with the subset profile observed in these experiments.

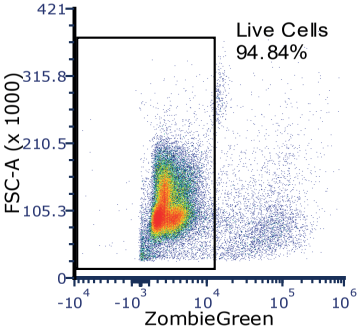
Equal variance of samples was assessed with a Brown-Forsythe statistical test and normal distribution with a Shapiro-Wilk test. Statistical significance between Cre MEF and iTEC conditions at sixteen weeks was tested with unpaired t tests for normally distributed data (αβ T cells, regulatory, γδ T cells and effector T cells) or a Mann-Whitney statistical test for non-parametric data (SP4, antigen experienced T cells). SP8, naïve and naïve and central memory T cells did not display equal variance and were therefore not statistically tested.

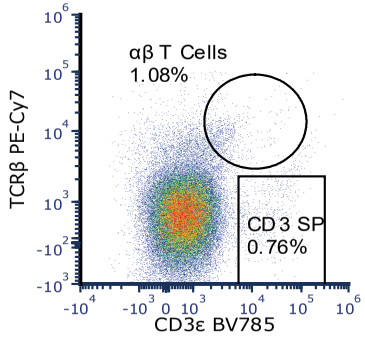
Statistical analysis revealed αβ T cells (p=0.012), SP4 T cells (p=0.0061) and antigen experienced T cells (p=0.0061) were significantly upregulated in iTEC recipients in comparison to negative control Cre MEFs at sixteen weeks post-transplantation. At four-weeks post transplantation, only SP8 and naïve T cells had equal variance but were not significantly different between iTEC and Cre MEF conditions.

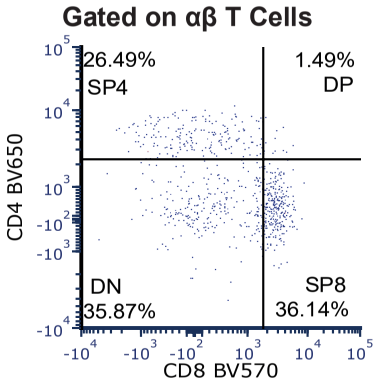
Despite the differences in absolute cell numbers (Tables 6.3 and 6.4), RFTOC and iTEC-RTOC recipients has similar T cell subset distributions, with SP8s belonging predominantly to naïve and central memory subsets and SP4s to antigen experienced T cells. Ki67 expression revealed that a subset of SP4s highly expressed Ki67 in both RFTOC and iTEC-RTOC recipients, and is therefore proliferating, in line with the fact they exhibited an antigen experienced phenotype, while SP8s showed intermediate levels of Ki67 (Figures 6.8 - 6.13).

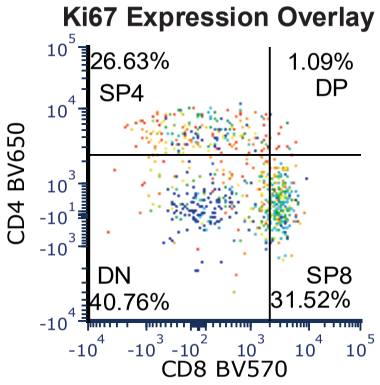

SSC-H (x 1000)
2983
2212.4
1441.8
671.2
-99.4
Cells
80.41%
0
295.2 590.4 885.5
FSC-H (x 1000)

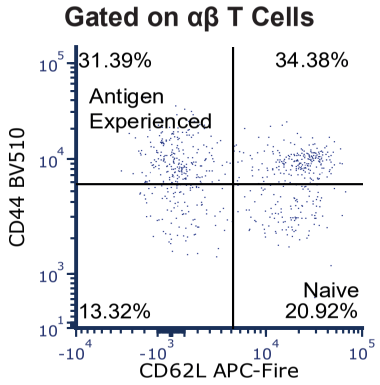

FSC-A (x 1000)
580.8
429.8
278.8
127.8
-23.2
Single Cells
98.94%
0
338.1 676.2 1014.2
FSC-H (x 1000)


FSC-A (x 1000)
421
315.8
210.5
105.3
0
Live Cells
94.84%
ZombieGreen


TCRβ PE-Cy7
αβ T Cells
1.08%
CD3 SP
0.76%
CD3ε BV785


Gated on αβ T Cells
26.49%
SP4
1.49%
DP
DN
35.87%
SP8
36.14%
CD4 BV650
CD8 BV570


Ki67 Expression Overlay
26.63%
SP4
1.09%
DP
DN
40.76%
SP8
31.52%
CD4 BV650
CD8 BV570


Gated on αβ T Cells
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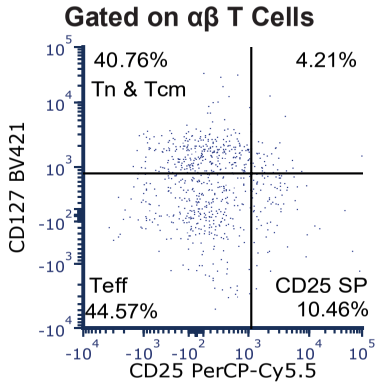
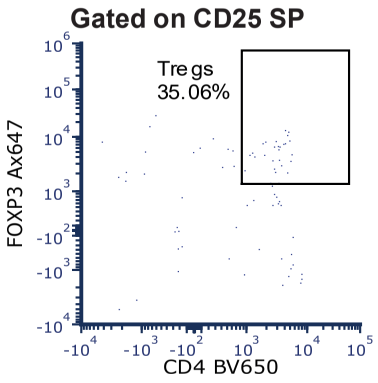
34.38%
Antigen
Experienced
13.32%
Naive
20.92%
CD44 BV510
CD62L APC-Fire


Gated on αβ T Cells
40.76%
4.21%
Tn & Tcm
Teff
44.57%
CD25 SP
10.46%
CD127 BV421
CD25 PerCP-Cy5.5


Gated on CD25 SP
Tregs
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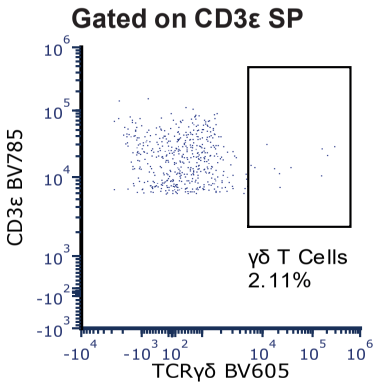
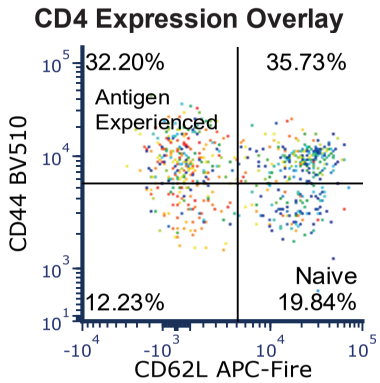
FOXP3 Ax647
CD4 BV650


Gated on CD3ε SP
γδ T Cells
2.11%
CD3ε BV785
TCRγδ BV605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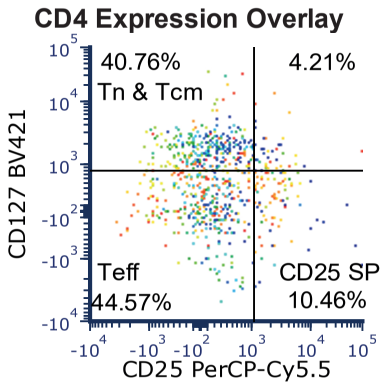
35.73%
Antigen
Experienced
12.23%
Naive
19.84%
CD44 BV510
CD62L APC-Fire


CD4 Expression Overlay
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Tn & Tcm
Teff
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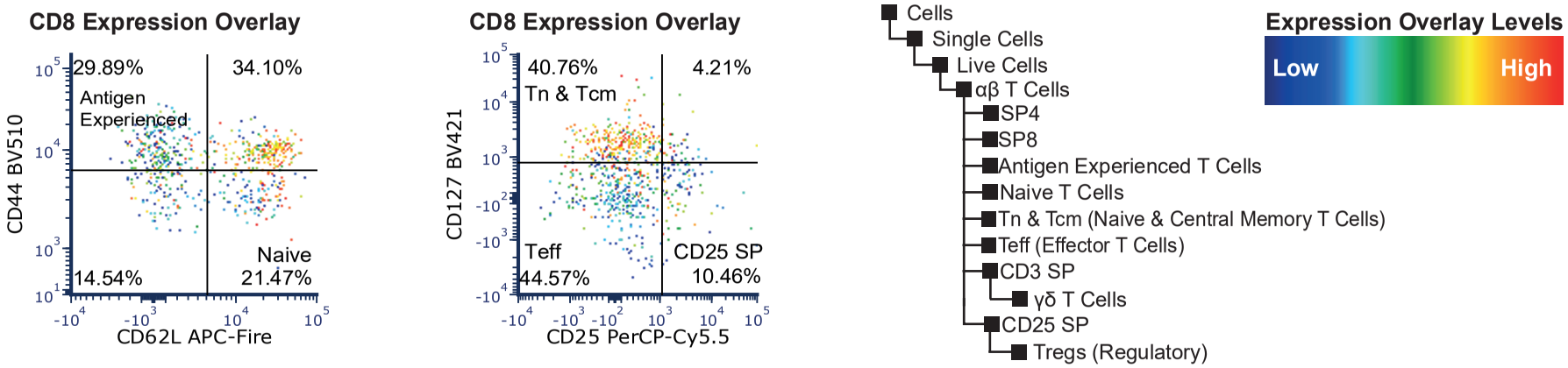
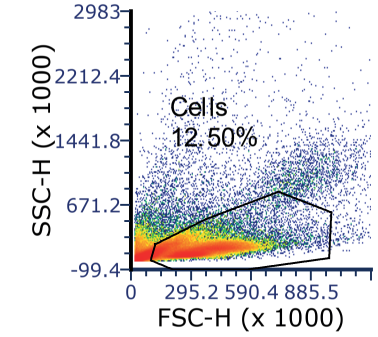


Figure 6.8. RFTOC recipient peripheral blood T cell analysis: 4 weeks post-transplantation. Representative FACS plots and gating hierarchy for RFTOC recipient peripheral blood 4 weeks post transplantation (animal ID 429). *Foxn1*$^{G/G}$ transplant recipients received one RFTOC consisting of six dissociated whole E15.5 thymic lobes + 200,000 WT MEFs.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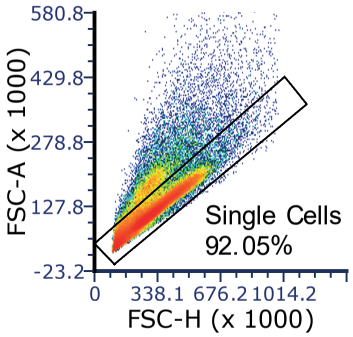
671.2
-99.4
Cells
12.50%
0
295.2 590.4 885.5
FSC-H (x 1000)


FSC-A (x 1000)
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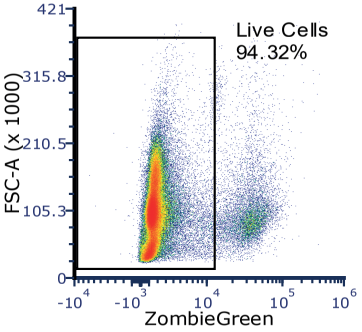
429.8
278.8
127.8
-23.2
Single Cells
92.05%
0
338.1 676.2 1014.2
FSC-H (x 1000)


FSC-A (x 1000)
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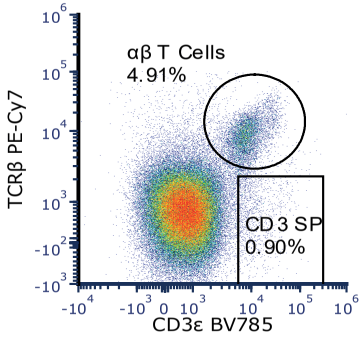
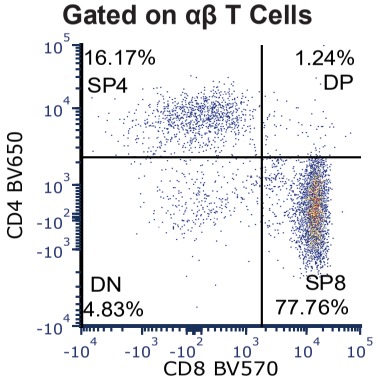
315.8
210.5
105.3
0
Live Cells
94.32%
ZombieGreen


TCRβ PE-Cy7
αβ T Cells
4.91%
CD3 SP
0.90%
CD3ε BV785


Gated on αβ T Cells
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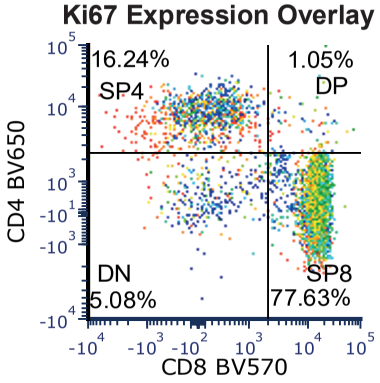
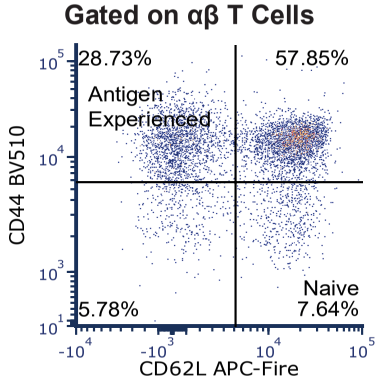
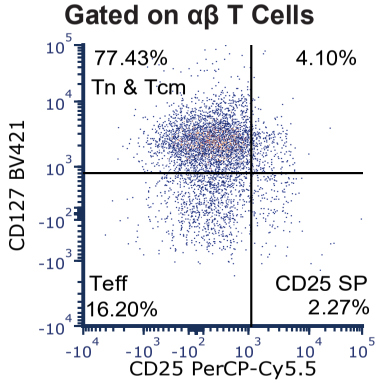
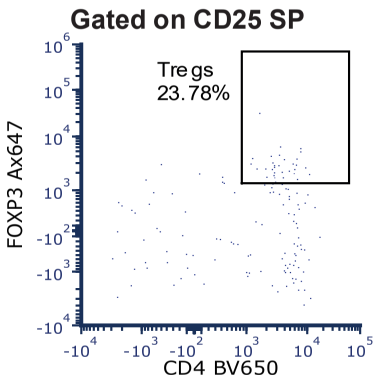
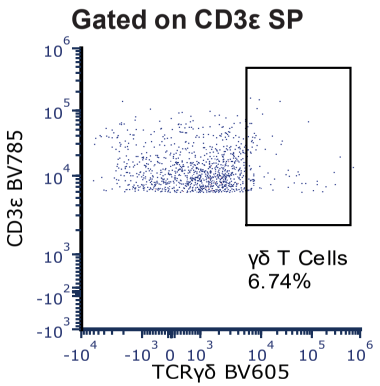
16.17%
SP4
1.24%
DP
DN
4.83%
SP8
77.76%
CD8 BV570

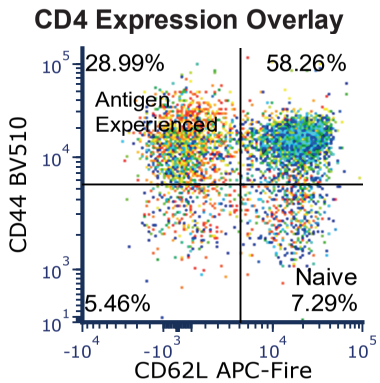

Ki67 Expression Overlay
CD4 BV650
16.24%
SP4
1.05%
DP
DN
5.08%
SP8
77.63%
CD8 BV570

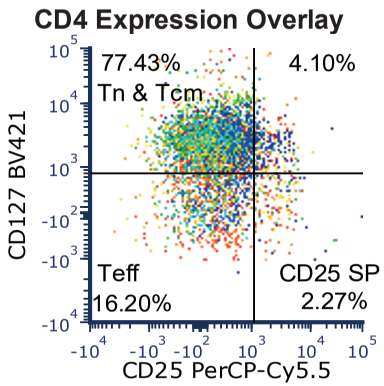

Gated on αβ T Cells
CD44 BV510
28.73%
57.85%
Antigen
Experienced
5.78%
Naive
7.64%
CD62L APC-Fire


Gated on αβ T Cells
CD127 BV421
77.43%
4.10%
Tn & Tcm
Teff
16.20%
CD25 SP
2.27%
CD25 PerCP-Cy5.5


Gated on CD25 SP
FOXP3 Ax647
Tregs
23.78%
CD4 BV650


Gated on CD3ε SP
CD3ε BV785
γδ T Cells
6.74%
TCRγδ BV605


CD4 Expression Overlay
CD44 BV510
28.99%
58.26%
Antigen
Experienced
5.46%
Naive
7.29%
CD62L APC-Fire


CD4 Expression Overlay
CD127 BV421
77.43%
4.10%
Tn & Tcm
Teff
16.20%
CD25 SP
2.27%
CD25 PerCP-Cy5.5

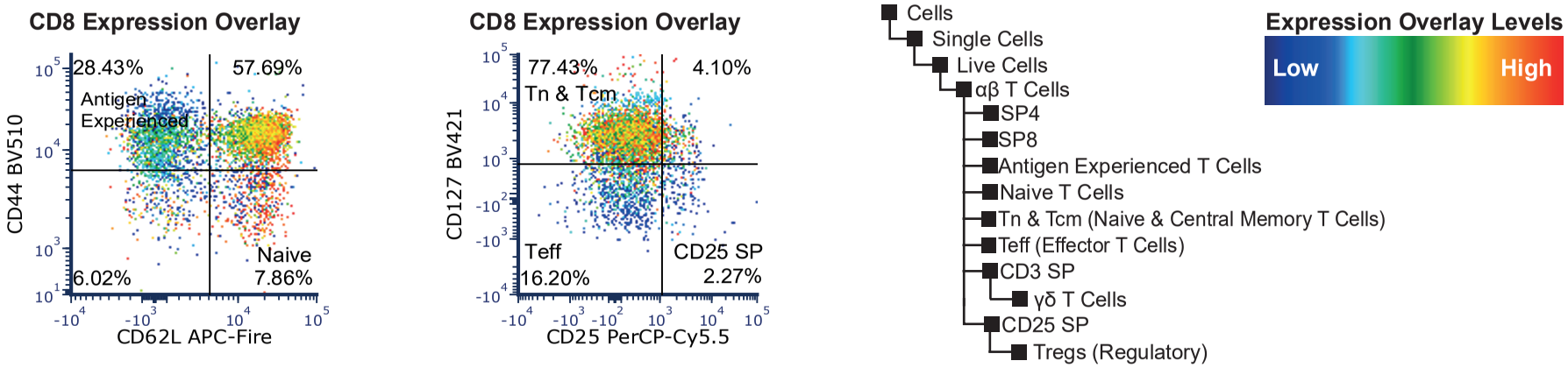
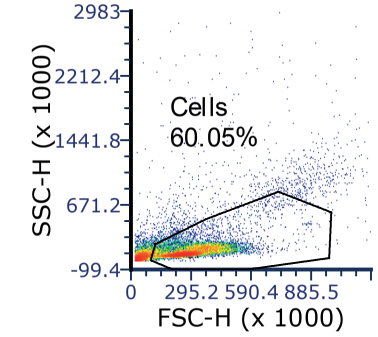
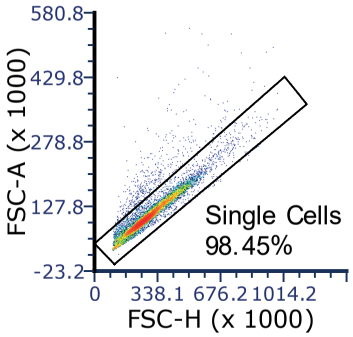
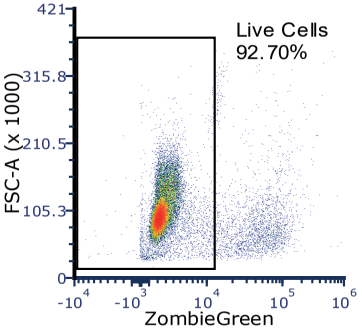
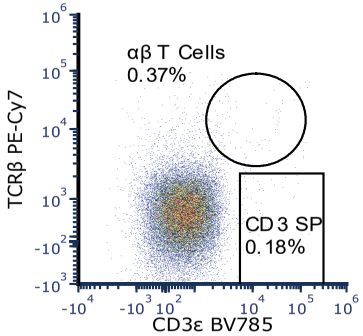


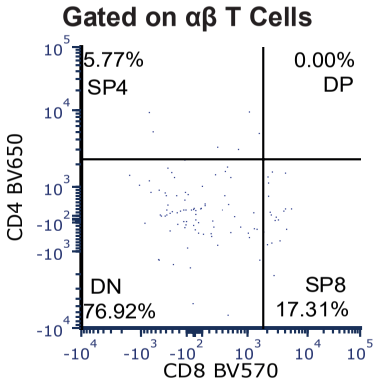
Figure 6.9. RFTOC recipient peripheral blood T cell analysis: 16 weeks post-transplantation. Representative FACS plots and gating hierarchy for RFTOC recipient peripheral blood 16 weeks post transplantation (animal ID 429). *Foxn1*$^{G/G}$ transplant recipients received one RFTOC consisting of six dissociated whole E15.5 thymic lobes + 200,000 WT MEFs.

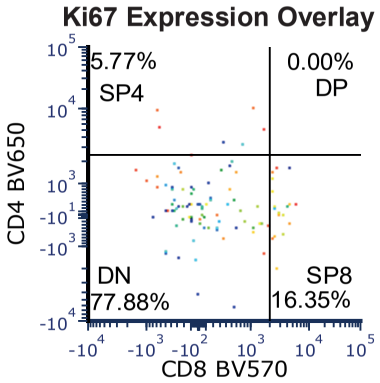

Cells
60.05%
SSC-H (x 1000)
FSC-H (x 1000)

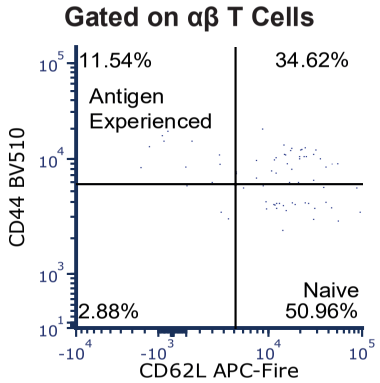

Single Cells
98.45%
FSC-A (x 1000)
FSC-H (x 1000)

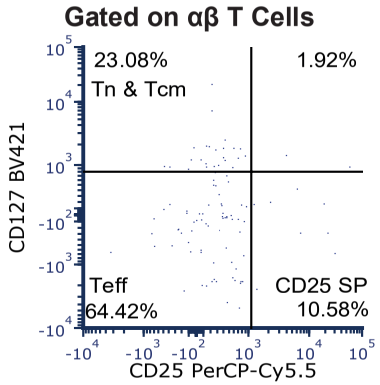

Live Cells
92.70%
FSC-A (x 1000)
ZombieGreen

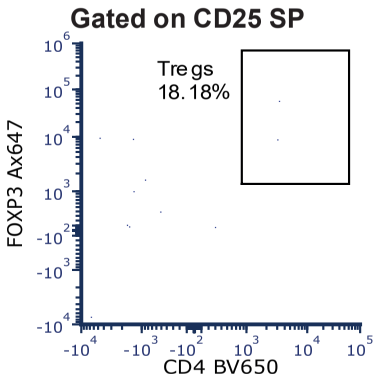

αβ T Cells
0.37%
CD3 SP
0.18%
TCRβ PE-Cy7
CD3ε BV785

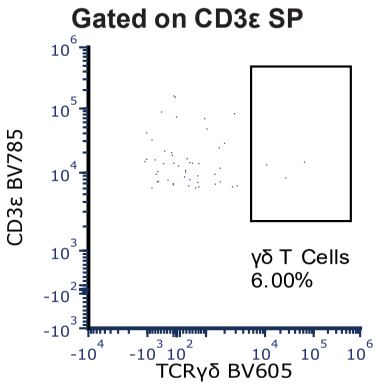

Gated on αβ T Cells
5.77%
SP4
0.00%
DP
DN
76.92%
SP8
17.31%
CD4 BV650
CD8 BV570

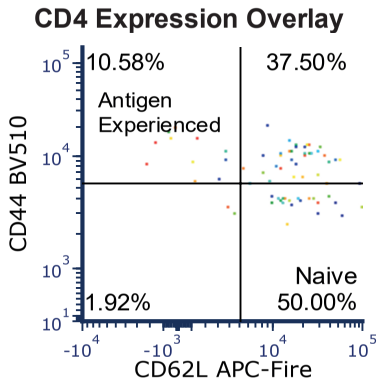

Ki67 Expression Overlay
5.77%
SP4
0.00%
DP
DN
77.88%
SP8
16.35%
CD4 BV650
CD8 BV570

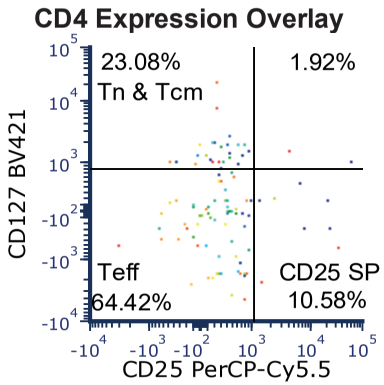

Gated on αβ T Cells
11.54%
34.62%
Antigen
Experienced
2.88%
Naive
50.96%
CD44 BV510
CD62L APC-Fire


Gated on αβ T Cells
23.08%
1.92%
Tn & Tcm
Teff
64.42%
CD25 SP
10.58%
CD127 BV421
CD25 PerCP-Cy5.5


Gated on CD25 SP
Tregs
18.18%
FOXP3 Ax647
CD4 BV650


Gated on CD3ε SP
γδ T Cells
6.00%
CD3ε BV785
TCRγδ BV605


CD4 Expression Overlay
10.58%
37.50%
Antigen
Experienced
1.92%
Naive
50.00%
CD44 BV510
CD62L APC-Fire


CD4 Expression Overlay
23.08%
1.92%
Tn & Tcm
Teff
64.42%
CD25 SP
10.58%
CD127 BV421
CD25 PerCP-Cy5.5

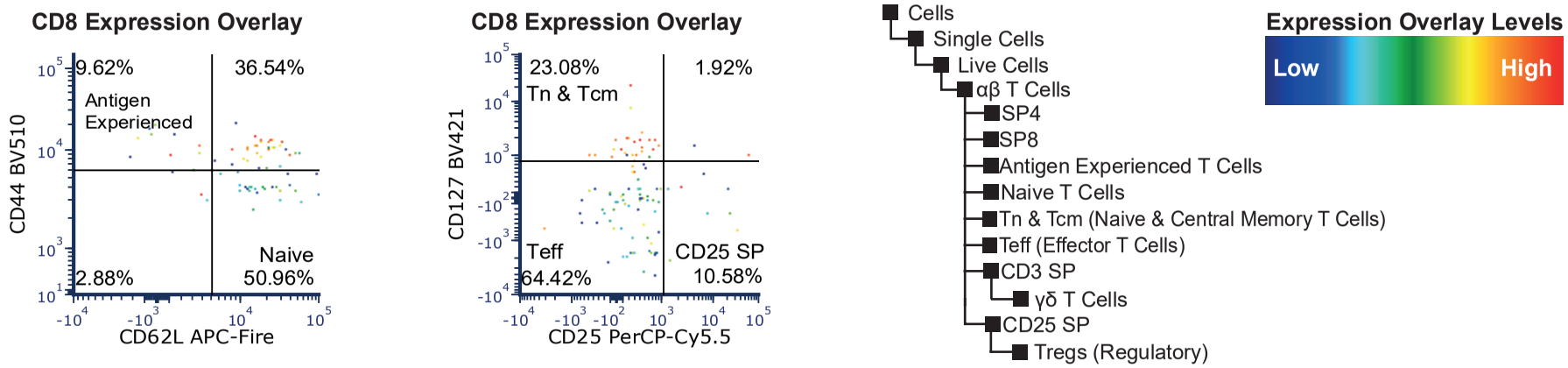
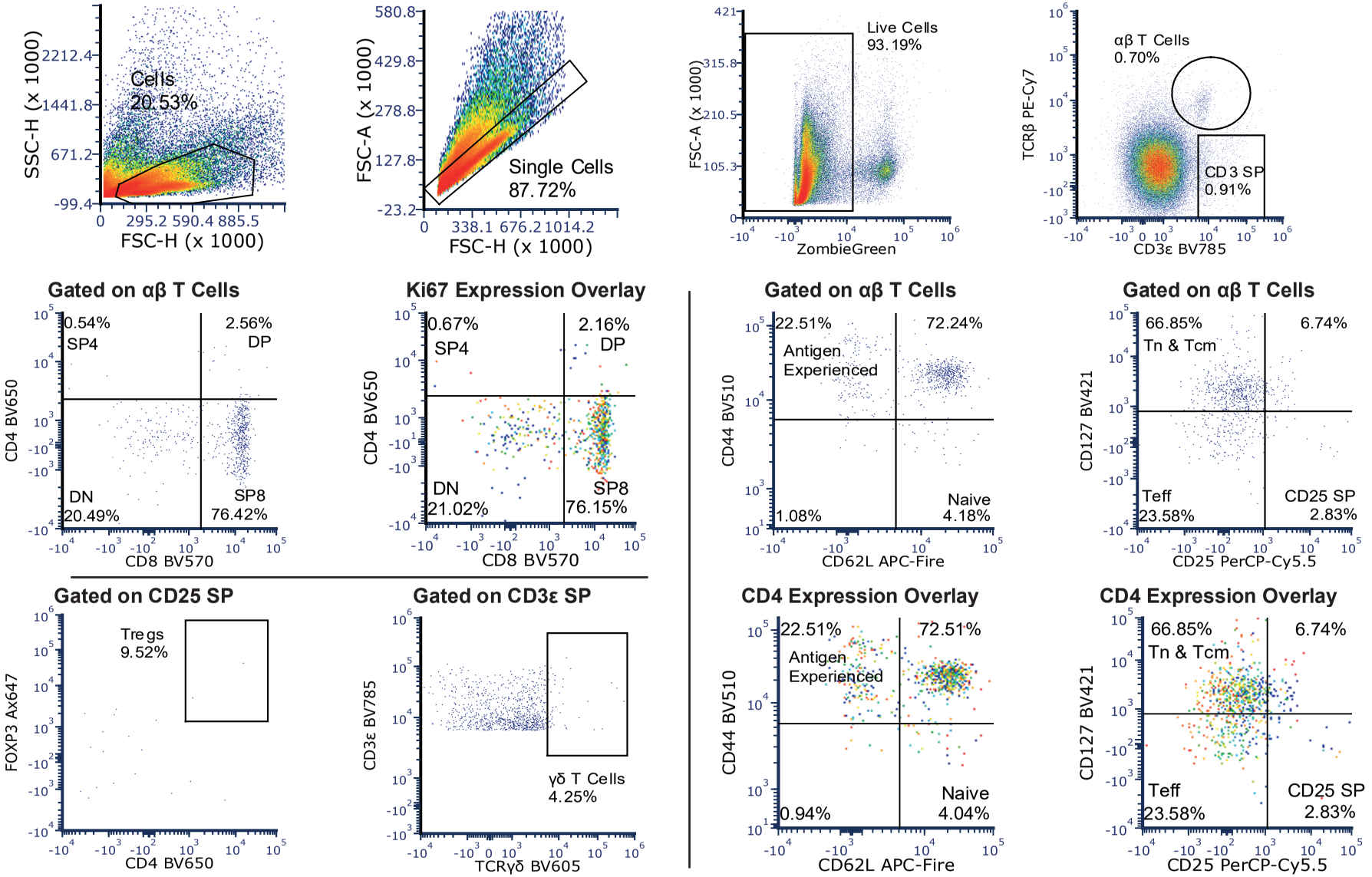


Figure 6.10. Cre MEF-MultiRTOC recipient peripheral blood T cell analysis: 4 weeks post-transplantation. Representative FACS plots and gating hierarchy for Cre MEF recipient peripheral blood 4 weeks post transplantation (animal ID 356). *Foxn1*$^{G/G}$ transplant recipients received three RTOC each consisting of 600,000 Cre MEFs + 100,000 DN Thymocytes +100,000 WT MEFs.


Cells
20.53%
SSC-H (x 1000)
FSC-H (x 1000)
Single Cells
87.72%
FSC-A (x 1000)
FSC-H (x 1000)
Live Cells
93.19%
FSC-A (x 1000)
ZombieGreen
αβ T Cells
0.70%
CD3 SP
0.91%
TCRβ PE-Cy7
CD3ε BV785
Gated on αβ T Cells
0.54%
SP4
2.56%
DP
DN
20.49%
SP8
76.42%
CD4 BV650
CD8 BV570
Ki67 Expression Overlay
0.67%
SP4
2.16%
DP
DN
21.02%
SP8
76.15%
CD4 BV650
CD8 BV570
Gated on αβ T Cells
22.51%
Antigen
Experienced
72.24%
1.08%
Naive
4.18%
CD44 BV510
CD62L APC-Fire
Gated on αβ T Cells
66.85%
Tn & Tcm
6.74%
Teff
23.58%
CD25 SP
2.83%
CD127 BV421
CD25 PerCP-Cy5.5
Gated on CD25 SP
Tregs
9.52%
FOXP3 Ax647
CD4 BV650
Gated on CD3ε SP
γδ T Cells
4.25%
CD3ε BV785
TCRγδ BV605
CD4 Expression Overlay
22.51%
Antigen
Experienced
72.51%
0.94%
Naive
4.04%
CD44 BV510
CD62L APC-Fire
CD4 Expression Overlay
66.85%
Tn & Tcm
6.74%
Teff
23.58%
CD25 SP
2.83%
CD127 BV421
CD25 PerCP-Cy5.5

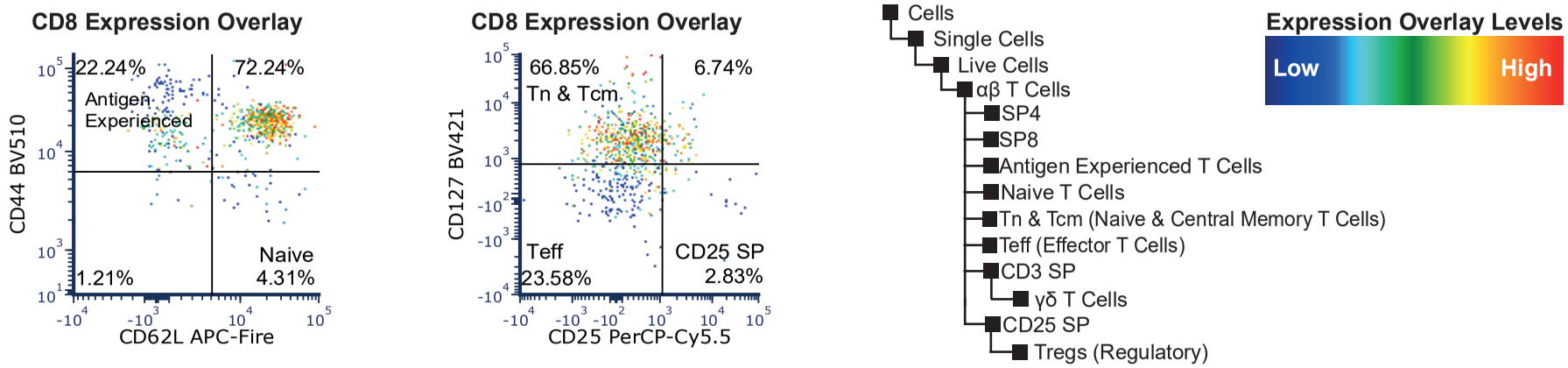
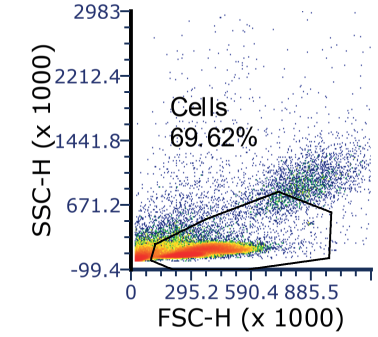
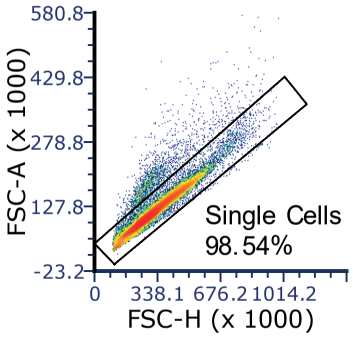
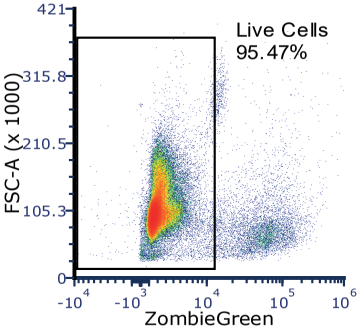
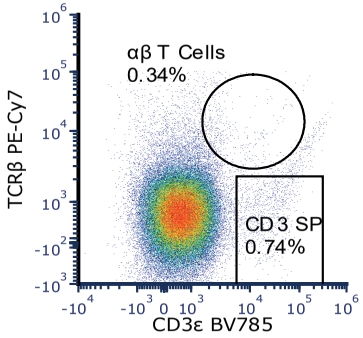


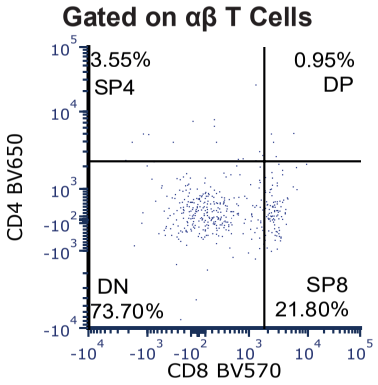
Figure 6.11. Cre MEF-MultiRTOC recipient peripheral blood T cell analysis: 16 weeks post-transplantation. Representative FACS plots and gating hierarchy for Cre MEF recipient peripheral blood 16 weeks post transplantation (animal ID 356). *Foxn1*$^{G/G}$ transplant recipients received three RTOC each consisting of 600,000 Cre MEFs + 100,000 DN Thymocytes +100,000 WT MEFs.

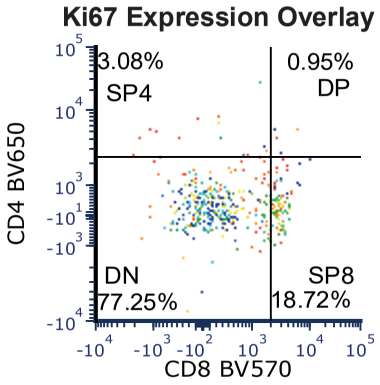

SSC-H (x 1000)
2983
2212.4
1441.8
671.2
-99.4
Cells
69.62%
0
295.2 590.4 885.5
FSC-H (x 1000)

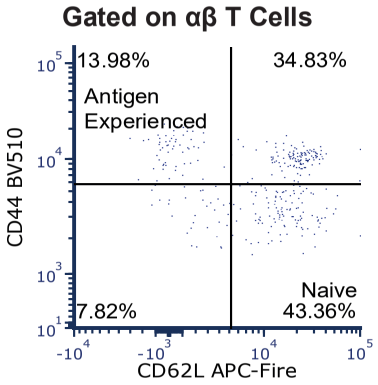

FSC-A (x 1000)
580.8
429.8
278.8
127.8
-23.2
Single Cells
98.54%
0
338.1 676.2 1014.2
FSC-H (x 1000)

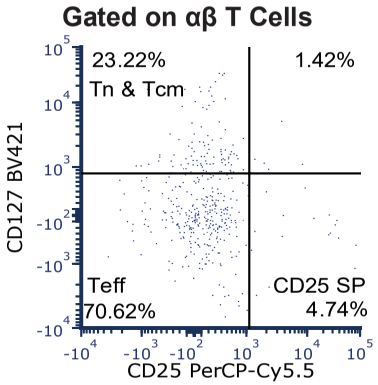

FSC-A (x 1000)
421
315.8
210.5
105.3
0
Live Cells
95.47%
ZombieGreen

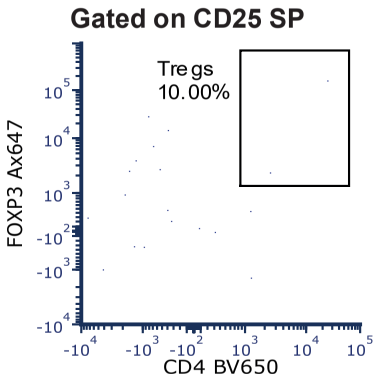

TCRβ PE-Cy7
αβ T Cells
0.34%
CD3 SP
0.74%
CD3ε BV785

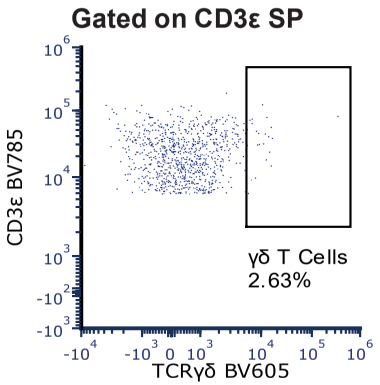

Gated on αβ T Cells
CD4 BV650
3.55%
SP4
0.95%
DP
DN
73.70%
SP8
21.80%
CD8 BV570

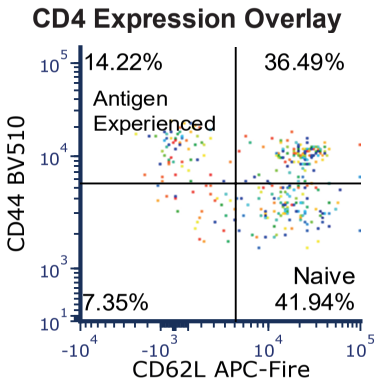

Ki67 Expression Overlay
CD4 BV650
3.08%
SP4
0.95%
DP
DN
77.25%
SP8
18.72%
CD8 BV570

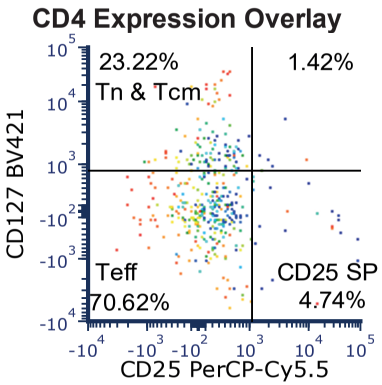

Gated on αβ T Cells
CD44 BV510
13.98%
34.83%
Antigen
Experienced
7.82%
Naive
43.36%
CD62L APC-Fire


Gated on αβ T Cells
CD127 BV421
23.22%
Tn & Tcm
1.42%
Teff
70.62%
CD25 SP
4.74%
CD25 PerCP-Cy5.5


Gated on CD25 SP
FOXP3 Ax647
Tregs
10.00%
CD4 BV650


Gated on CD3ε SP
CD3ε BV785
γδ T Cells
2.63%
TCRγδ BV605


CD4 Expression Overlay
CD44 BV510
14.22%
36.49%
Antigen
Experienced
7.35%
Naive
41.94%
CD62L APC-Fire


CD4 Expression Overlay
CD127 BV421
23.22%
Tn & Tcm
1.42%
Teff
70.62%
CD25 SP
4.74%
CD25 PerCP-Cy5.5

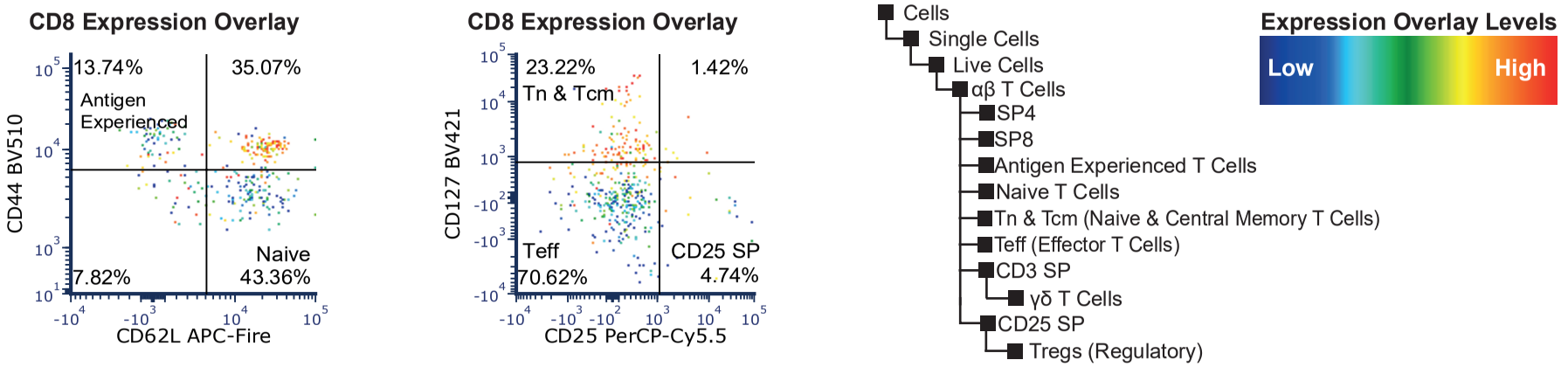
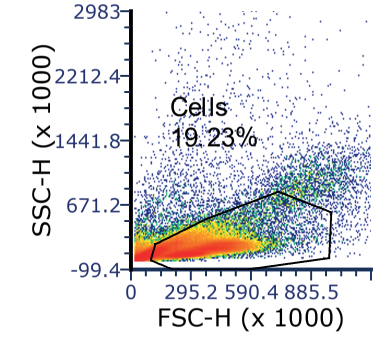
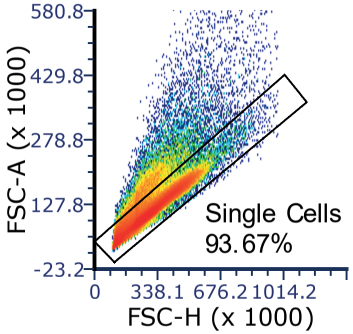
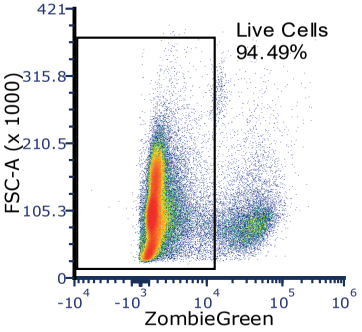
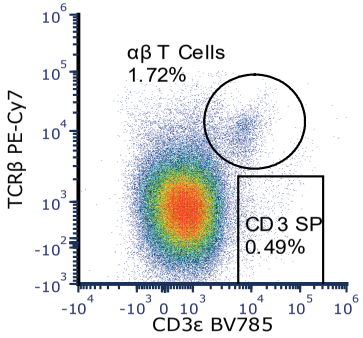


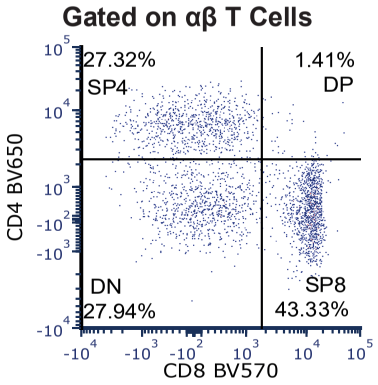
Figure 6.12. iTEC-MultiRTOC recipient peripheral blood T cell analysis: 4 weeks post-transplantation. Representative FACS plots and gating hierarchy for iTEC recipient peripheral blood 4 weeks post transplantation (animal ID 350). *Foxn1*$^{G/G}$ transplant recipients received three RTOC each consisting of 600,000 iTEC + 100,000 DN Thymocytes +100,000 WT MEFs.

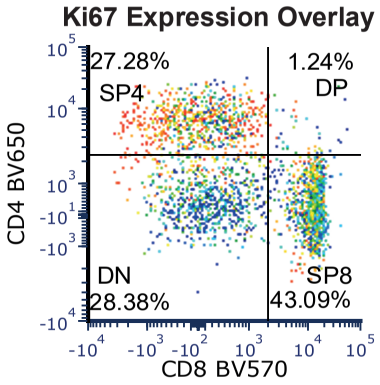

SSC-H (x 1000)
2983
2212.4
1441.8
671.2
-99.4
Cells
19.23%
0
295.2 590.4 885.5
FSC-H (x 1000)

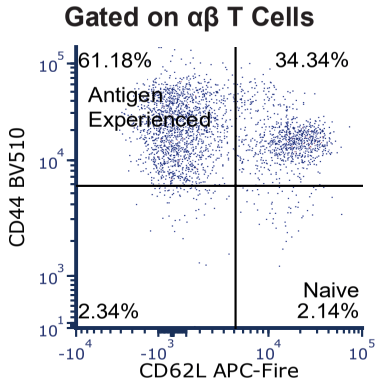

FSC-A (x 1000)
580.8
429.8
278.8
127.8
-23.2
Single Cells
93.67%
0
338.1 676.2 1014.2
FSC-H (x 1000)

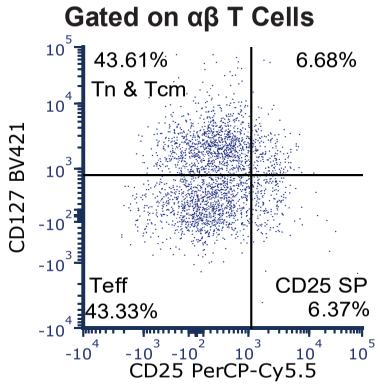

FSC-A (x 1000)
421
315.8
210.5
105.3
0
Live Cells
94.49%
ZombieGreen

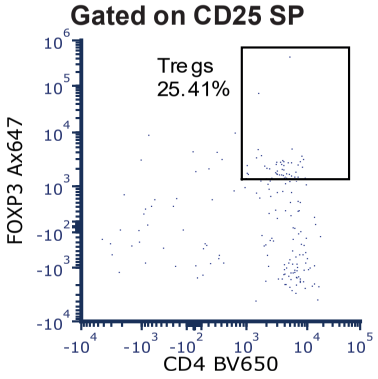

TCRβ PE-Cy7
αβ T Cells
1.72%
CD3 SP
0.49%
CD3ε BV785

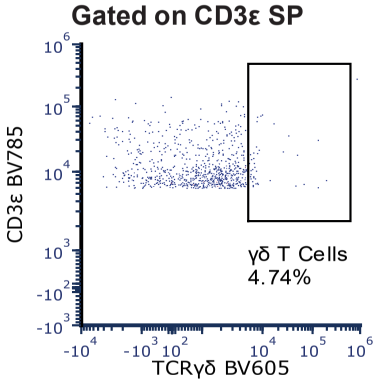

Gated on αβ T Cells
CD4 BV650
27.32%
SP4
1.41%
DP
DN
27.94%
SP8
43.33%
CD8 BV570

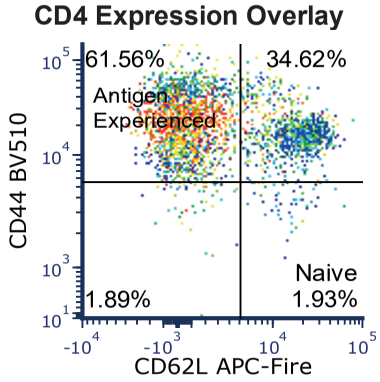

Ki67 Expression Overlay
CD4 BV650
27.28%
SP4
1.24%
DP
DN
28.38%
SP8
43.09%
CD8 BV570

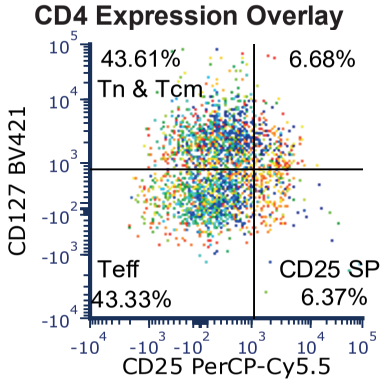

Gated on αβ T Cells
CD44 BV510
61.18%
34.34%
Antigen
Experienced
2.34%
Naive
2.14%
CD62L APC-Fire


Gated on αβ T Cells
CD127 BV421
43.61%
6.68%
Tn & Tcm
Teff
43.33%
CD25 SP
6.37%
CD25 PerCP-Cy5.5


Gated on CD25 SP
FOXP3 Ax647
Tregs
25.41%
CD4 BV650


Gated on CD3ε SP
CD3ε BV785
γδ T Cells
4.74%
TCRγδ BV605


CD4 Expression Overlay
CD44 BV510
61.56%
34.62%
Antigen
Experienced
1.89%
Naive
1.93%
CD62L APC-Fire


CD4 Expression Overlay
CD127 BV421
43.61%
6.68%
Tn & Tcm
Teff
43.33%
CD25 SP
6.37%
CD25 PerCP-Cy5.5

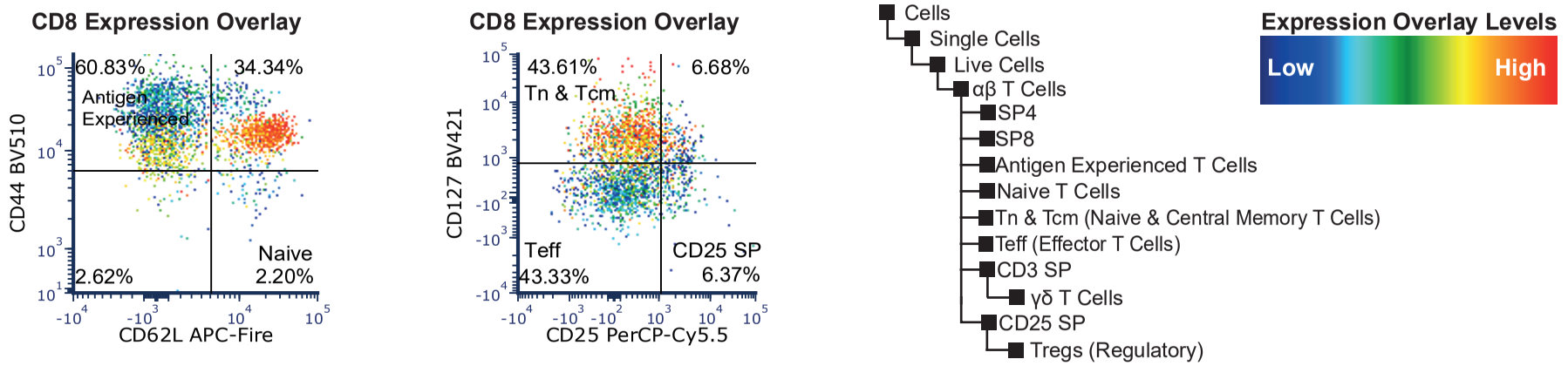


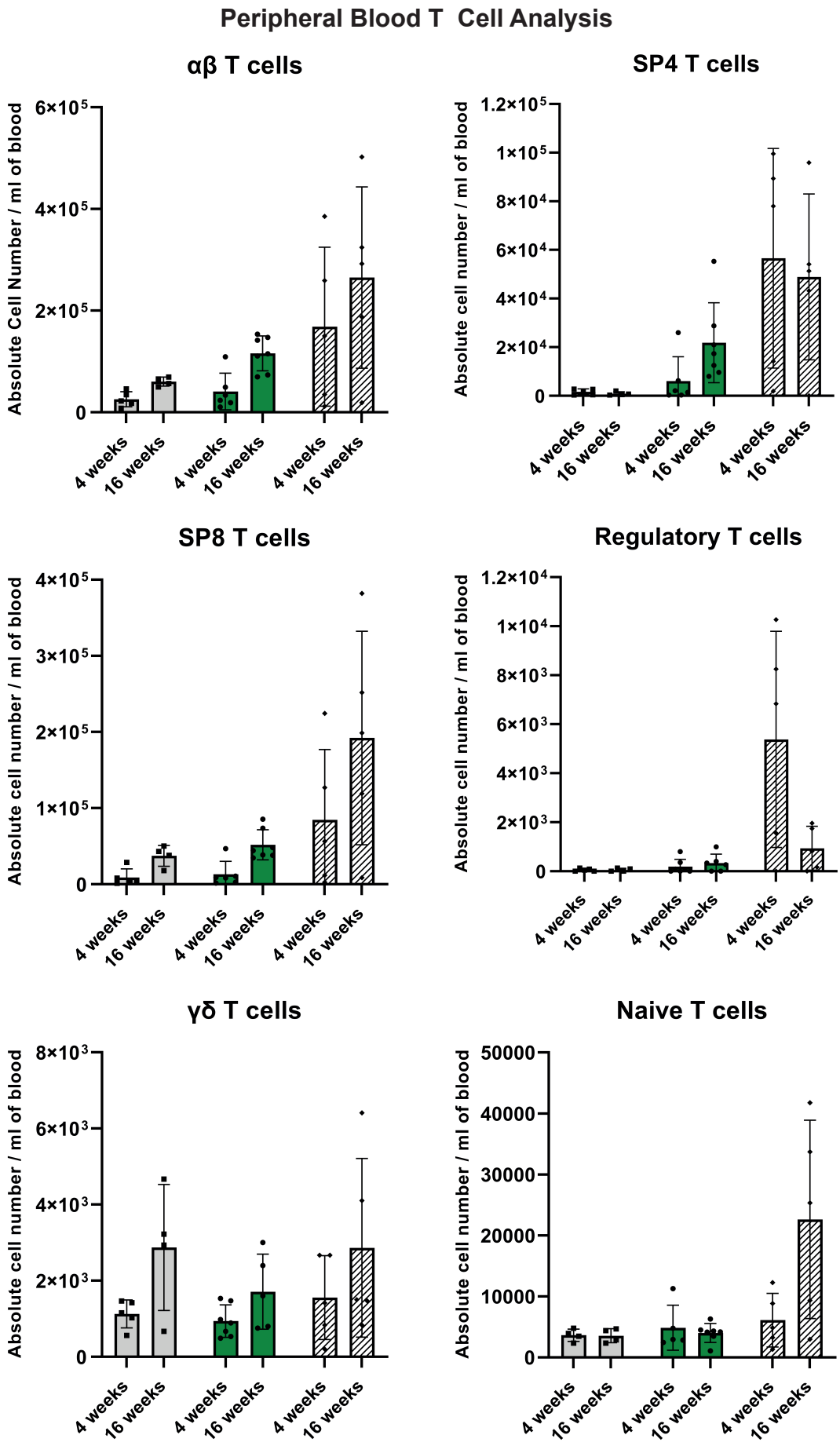
Figure 6.13. iTEC-MultiRTOC recipient peripheral blood T cell analysis: 16 weeks post-transplantation. Representative FACS plots and gating hierarchy for iTEC recipient peripheral blood 16 weeks post transplantation (animal ID 350). *Foxn1*$^{G/G}$ transplant recipients received three RTOC each consisting of 600,000 iTEC + 100,000 DN Thymocytes +100,000 WT MEFs.

Condition	Surgery Rep	Animal ID	αβ T cells								CD3 SP
			Total	SP4	SP8	Regulatory	Naïve	Naïve & central memory	Effector memory	Antigen Experienced	γδ T cells
Cre MEFs	Rep A	**356**	34627	2667	7957	44		5601	26937	6134	1156
	Rep B	**416**	45999	2000	28666	133	4800	25066	17333	10266	1467
	Rep B	**486**	17600	2756	4000	89	3467	3644	12267	9067	1422
	Rep C	**500**	22454	421	3228	0	3930	2947	17893	13052	561
	Rep C	**530**	7795	308	1231	0	2359	1128	5846	2872	1026
		Mean	**25695 ± 13330**	**1630 ± 1066**	**9016 ± 10064**	**53 ± 51**	**3639 ± 880**	**7677 ± 8811**	**16055 ± 6957**	**8278 ± 3498**	**1126 ± 326**
iTEC	Rep A	**321**	109246	25956	46845	800		42312	54667	39645	978
	Rep A	**350**				356			155131	33204	533
	Rep A	***315**	33868	6267	8867	0	11267	13734	17401	9000	667
	Rep B	**455**	23645	1422	10578	89	2844	7822	11378	15911	889
	Rep B	***501**	10534	267	1600	0	2467	1600	7867	4067	1533
	Rep C	**494**	49407	2035	8351	70	4772	5965	39792	31932	1474
	Rep C	**495**	19019	281	2807	0	2948	1544	15791	12562	491
		Mean	**40953 ± 32893**	**6038 ± 9134**	**13175 ± 15404**	**188 ± 276**	**4860 ± 3302**	**12163 ± 14101**	**43147 ± 48329**	**20903 ± 12783**	**938 ± 393**
RFTOC	Rep A	**429**	11801	1867	2667	0	4867	2267	8667	2667	200
	Rep B	**411**	150161	77997	56331	8250	3250	64997	34999	114245	1417
	Rep B	**417**	259060	89331	127063	10266	12266	115864	75598	160796	2667
	Rep C	**493**	385174	99502	224504	6833	8834	133669	133836	299339	833
	Rep C	**499**	35111	14222	11778	1556	1333	8000	13555	24666	2667
		Mean	**168261 ± 139879**	**56584 ± 40401**	**84469 ± 82693**	**5381 ± 3944**	**6110 ± 3946**	**64959 ± 53827**	**53331 ± 46668**	**120343 ± 106503**	**1557 ± 984**

Table 6.3. Absolute cell numbers of peripheral blood T cells at 4 weeks post-transplantation. Outliers were detected using the ROUT method and Q=1% setting in GraphPad Prism and removed from the analysis. Mean ± SD.

Condition	Surgery Rep	Animal ID	αβ T cells								CD3 SP
			Total	SP4	SP8	Regulatory	Naïve	Naïve & central memory	Effector	Antigen Experienced	γδ T cells
Cre MEFs	Rep A	**356**	49602	333	38135	133	2867	33135	11734	9934	2933
	Rep B	**416**	69222	444	50111	0	2333	49555	15444	14667	3222
	Rep B	**486**	65525	762	43048	95	4381	45049	18381	19048	
	Rep C	**500**									667
	Rep C	**530**	56667	2000	18000	0	4667	19333	37334	31333	4667
		Mean	**60254 ± 7657**	**884 ± 662**	**37323 ± 11940**	**57 ± 58**	**3562 ± 985**	**36768 ± 11718**	**20723 ± 9875**	**18745 ± 7950**	**2872 ± 1432**
iTEC	Rep A	**321**	141540	12534	85471	267	4200	69270	60470	79004	2400
	Rep A	**350**	110833	28804	48044		4153	48349	48006	60122	1600
	Rep A	***315**	147336	8000	38667	333	4500	58168	82335	96002	
	Rep B	**455**	115188	20976	73593	400	6310	59416	45507	67149	755
	Rep B	***501**	73332	9600	44132	0	1067	42132	26666	57065	
	Rep C	**494**	69601	17334	37867	0	3467	41067	25600	51467	800
	Rep C	**495**	153665	55333	34666	1000	4333	66333	72999	126999	3000
		Mean	**115928 ± 31744**	**21797 ± 15209**	**51777 ± 18290**	**333 ± 336**	**4004 ± 1447**	**54962 ± 10458**	**51655 ± 10458**	**76830 ± 24773**	**1711 ± 882**
RFTOC	Rep A	**429**	324358	51290	251885	1744	41750	251013	52624	74371	4103
	Rep B	**411**	502207	95852	382005	1965	25331	406144	76766	236683	1474
	Rep B	**417**	292040	54152	198761	820	33691	208556	64203	103637	6410
	Rep C	**493**	18667	0	8667	0	3000	8500	9334	6833	1500
	Rep C	**499**	187504	43168	119169	167	9334	140003	43001	91835	833
		Mean	**264955 ± 159503**	**48892 ± 30534**	**192097 ± 125418**	**939 ± 799**	**22621 ± 14541**	**202843 ± 130692**	**49186 ± 22910**	**102672 ± 74917**	**2864 ± 2098**

Table 6.4. Absolute cell numbers of peripheral blood T cells at 16 weeks post-transplantation. Outliers were detected using the ROUT method and Q=1% setting in GraphPad Prism and removed from the analysis. Mean ± SD.


Peripheral Blood T Cell Analysis
αβ T cells
Absolute Cell Number / ml of blood
6×10⁵
4×10⁵
2×10⁵
0
4 weeks
16 weeks
SP4 T cells
Absolute cell number / ml of blood
1.2×10⁵
1×10⁵
8×10⁴
6×10⁴
4×10⁴
2×10⁴
SP8 T cells
4×10⁵
3×10⁵
2×10⁵
1×10⁵
Regulatory T cells
1.2×10⁴
1×10⁴
8×10³
6×10³
4×10³
2×10³
γδ T cells
8×10³
Naive T cells
50000
40000
30000
20000
10000

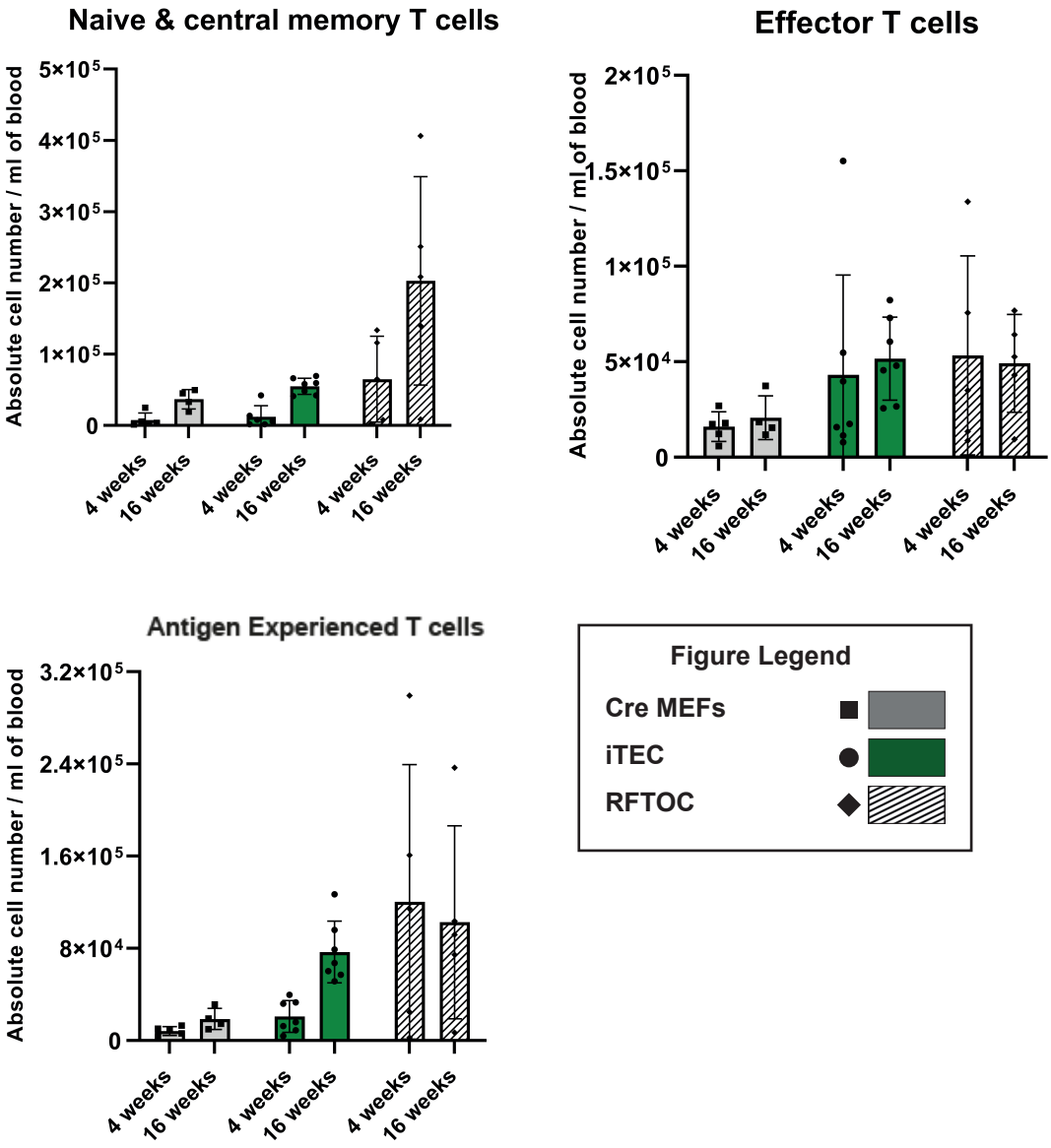


Figure 6.14. Absolute cell numbers per 1 ml of peripheral blood of T cells in *Foxn1*$^{G/G}$ transplanted mice at 4 and 16 weeks. Each datapoint represents one animal. αβ T cells (CD3ε$^+$TCRβ$^+$), SP4(CD4$^+$), SP8 (CD8$^+$), Regulatory (CD4$^+$FOXP3$^+$), γδ T cells (CD3ε$^+$TCRγδ$^+$), Naive (CD44$^-$CD62L$^+$), Naive & central memory (CD25$^-$CD127$^+$), Effector (CD25$^-$CD127$^-$), Antigen Experienced (CD44$^+$CD62L$^-$). Outliers were removed using the ROUT method and setting Q=1%. Absolute cell numbers were normalised depending on volume of sample obtained per animal at each timepoint to reflect numbers present in 1 ml of peripheral blood. Graphs display mean+SD. Cre MEFs 3 x (600,000 Cre MEFs + 100,000 WT MEFs + 100,000 DN Thymocytes), iTEC 3 x (600,000 iTEC + 100,000 WT MEFs + 100,000 DN Thymocytes), RFTOC (6 dissociated lobes + 200,000 WT MEFs). N=3 independent biological experiments.

6.2.B. Lymph node analysis

The gating strategy and representative FACS plots for the analysis of T cell numbers and subset distribution in the lymph nodes at the experimental endpoints are shown in Figures 6.15 – 6.19; the CD4/CD8 subset distribution is shown for every recipient (Figure 6.20). Upon microscopic examination of the thoracic cavity, no native thymus tissue was detected in any of the recipient *Foxn1*$^{G/G}$ mice.

The absolute numbers of lymph node cells were calculated and adjusted to represent the numbers found in a total of seven lymph nodes (2 axillary, 2 inguinal and 3 cervical lymph nodes). The absolute numbers of specific T cell populations (αβ, SP4, SP8, regulatory, γδ, naïve, naïve and central memory, effector and antigen experienced T cells) in the lymph nodes at twenty-two weeks post-transplantation are shown in Table 6.5, Figure 6.21.

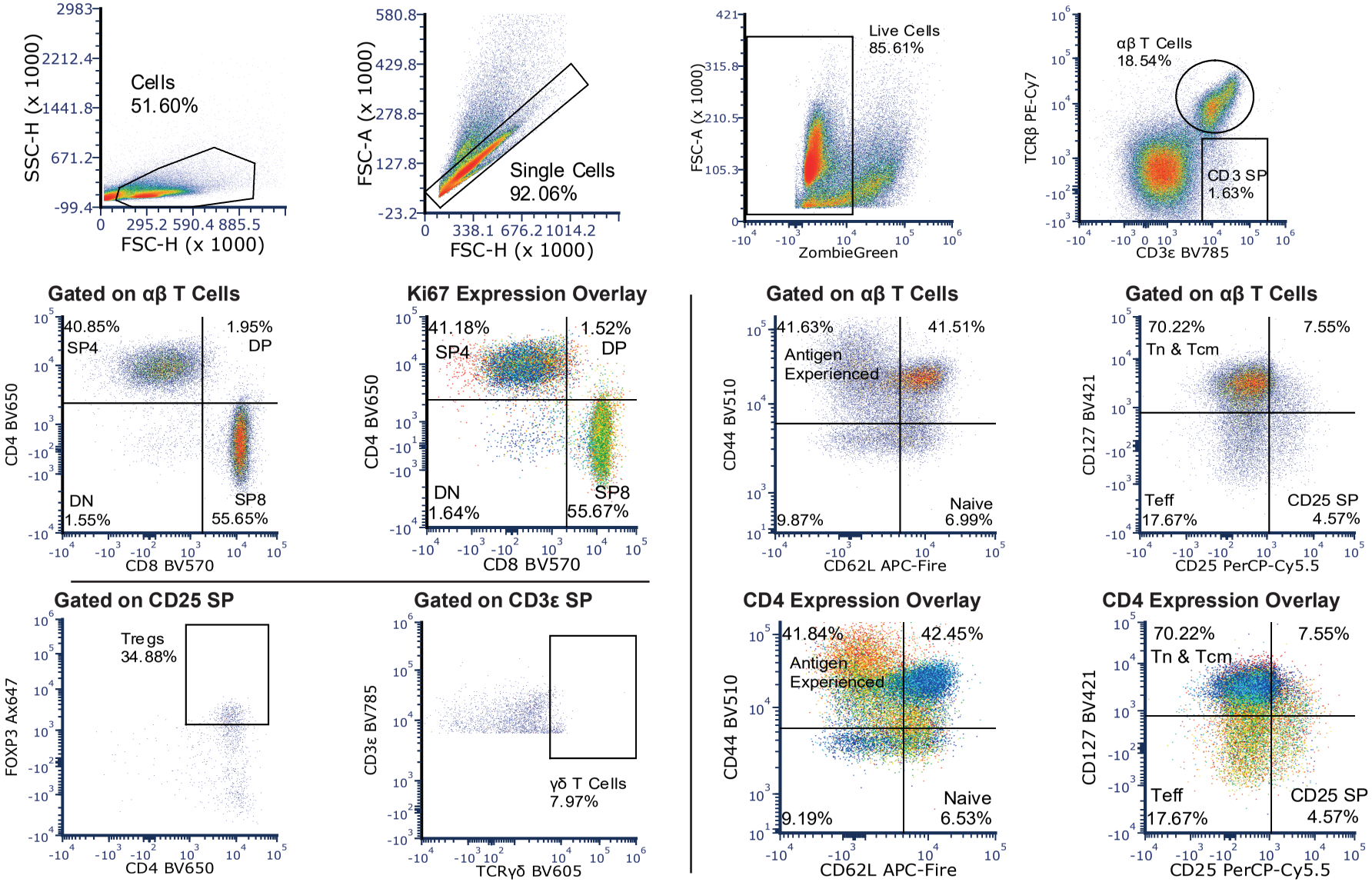
Of note is that two iTEC-RTOC recipient mice were culled at earlier timepoints, due to rapid weight loss that was most likely associated with autoimmunity. These were animals ID 315 and 501, which were culled at twenty and seventeen weeks, respectively. The likelihood that these mice had an infection is low as the facility is pathogen free and other mice in the same cage did not display any symptoms. These animals were included in the analysis to represent overall trends in T cell subsets in mice at experimental endpoints. The two sick mice had the lowest levels of regulatory, SP8, naïve and central memory and antigen experienced T cells, of all the iTEC recipients. No mice in either the negative or positive control groups became sick.

Analysis of total T cell numbers and the numbers of each subset examined demonstrated that the RFTOC recipients contained the highest numbers of cells in all T cell subsets, with the iTEC-RTOC recipients having higher numbers in all T cell subsets than the Cre MEF recipients. Equal variance (Brown-Forsythe) and normal distribution (Shapiro-Wilk) were tested for all samples as above. Statistical significance between Cre MEF and iTEC recipient conditions was tested with unpaired t tests for normally distributed data (αβ T cells, SP4s, SP8s, Tregs, γδ T cells, naïve & central memory and effector memory T cells) or a Mann-Whitney statistical test for non-parametric data (naïve, antigen experienced T cells). Subsequent statistical analysis revealed that the iTEC-RTOC recipients contained 2.8-fold more αβ ($p=0.0402$), 1.9-fold more γδ ($p=$ ns), 5.8-fold more SP4

(p=0.0196), 3-fold more effector memory (p=0.024), 30-fold more regulatory (0.0267) and 4.4-fold more antigen experienced T cells (p=0.0101) than the negative control Cre MEF recipients. The mean effector and antigen experienced T cell numbers present in the iTEC recipients (1.3×10^5 and 2×10^5) closely resembled those found in the RFTOC recipient mice (1.5×10^5 and 2.3×10^5).

Furthermore, expression overlay plots demonstrated that in the positive control samples, most SP4 T cells were in the antigen experienced and effector memory subsets, while SP8s were predominantly naïve and central memory phenotype cells. This pattern was also found in the iTEC-RTOC recipients, with the addition that a large proportion of SP4s were also naïve and central memory T cells. The iTEC recipients that became ill displayed more antigen experienced SP8 cells, and more SP4 that expressed high levels of Ki67. One possibility for the presence of more antigen experienced SP8s and proliferating SP4s in sick recipients would be antigen-specific proliferation resulting from an autoimmune response or infection.

Immunodeficiency is associated with an inverted (<1) CD4/CD8 ratio (Fahey *et al*., 2000; Izaks *et al*., 2003; Akbar and Fletcher, 2005; Shao *et al*., 2017). In order to determine immunodeficiency status in iTEC recipients, CD4:CD8 T cell ratios were also explored and compared to that of positive controls (Table 6.6). When investigating the CD4:CD8 T cell ratio of individual mice of all three conditions in lymph nodes of recipients, Cre MEF recipients averaged at 0.35, iTEC recipients at 1.28 and RFTOC recipients at 0.74. The low values of the CD4:CD8 T cell ratio in all Cre MEF recipients highlights the still present immunodeficient status of the animals. In contrast the higher values of the CD4:CD8 ratio (> 0.7) of the RFTOC recipients should indicate immunodeficiency reversal as is in line with thymic tissue transplantation. In agreement with this, iTEC recipients displayed CD4:CD8 ratio values over 0.7 with the exception of mouse 494 (0.56). Additionally, five out of seven iTEC recipients display values >1 suggesting immunodeficiency reversal.


Cells
51.60%
SSC-H (x 1000)
FSC-H (x 1000)
Single Cells
92.06%
FSC-A (x 1000)
FSC-H (x 1000)
Live Cells
85.61%
FSC-A (x 1000)
ZombieGreen
αβ T Cells
18.54%
CD3 SP
1.63%
TCRβ PE-Cy7
CD3ε BV785
Gated on αβ T Cells
40.85%
SP4
1.95%
DP
DN
1.55%
SP8
55.65%
CD4 BV650
CD8 BV570
Ki67 Expression Overlay
41.18%
SP4
1.52%
DP
DN
1.64%
SP8
55.67%
CD4 BV650
CD8 BV570
Gated on αβ T Cells
41.63%
41.51%
Antigen
Experienced
9.87%
Naive
6.99%
CD44 BV510
CD62L APC-Fire
Gated on αβ T Cells
70.22%
7.55%
Tn & Tcm
Teff
17.67%
CD25 SP
4.57%
CD127 BV421
CD25 PerCP-Cy5.5
Gated on CD25 SP
Tregs
34.88%
FOXP3 Ax647
CD4 BV650
Gated on CD3ε SP
γδ T Cells
7.97%
CD3ε BV785
TCRγδ BV605
CD4 Expression Overlay
41.84%
42.45%
Antigen
Experienced
9.19%
Naive
6.53%
CD44 BV510
CD62L APC-Fire
CD4 Expression Overlay
70.22%
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Tn & Tcm
Teff
17.67%
CD25 SP
4.57%
CD127 BV421
CD25 PerCP-Cy5.5

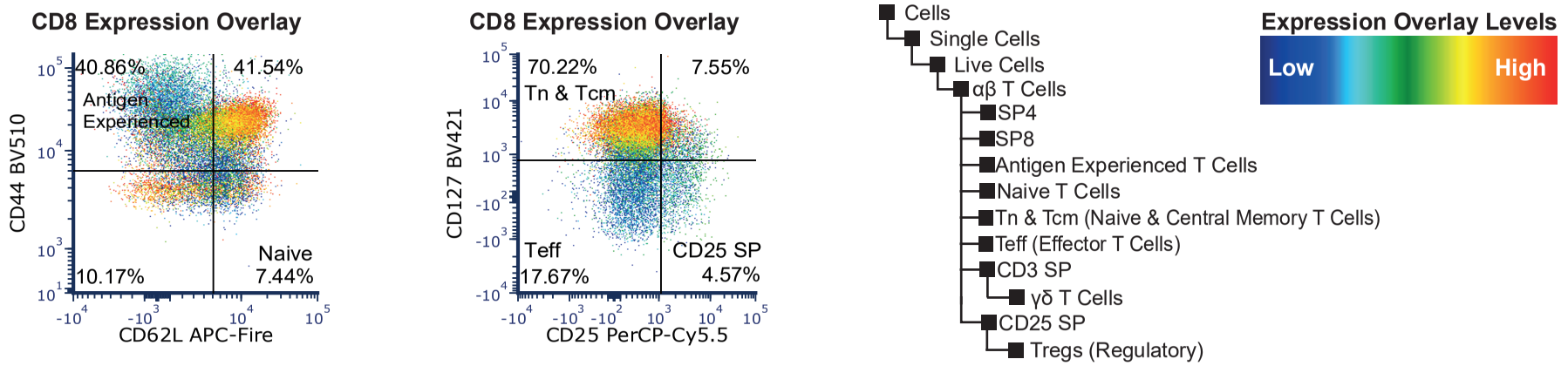
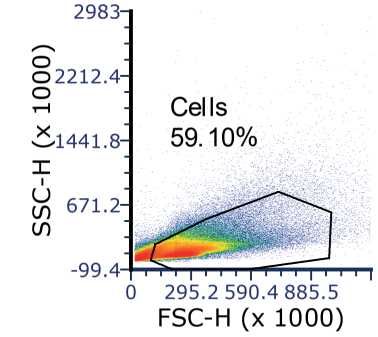
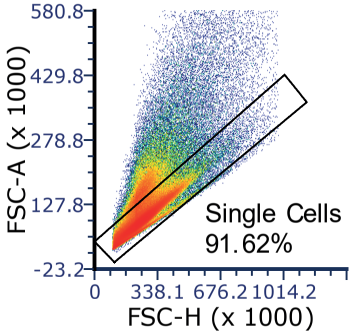
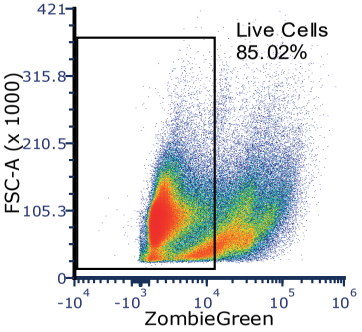
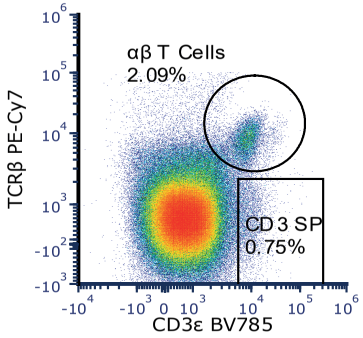


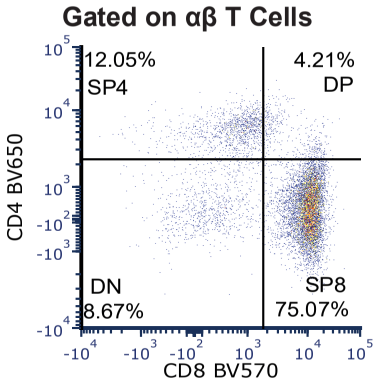
Figure 6.15. RFTOC recipient lymph node T cell analysis: 22 weeks post-transplantation. Representative FACS plots and gating hierarchy for RFTOC recipient lymph node T cells 22 weeks post transplantation (animal ID 499). Axillary, inguinal and cervical lymph nodes were collected. *Foxn1*$^{G/G}$ transplant recipients received one RFTOC each consisting of 6 dissociated whole E15.5 thymic lobes + 200,000 WT MEFs.

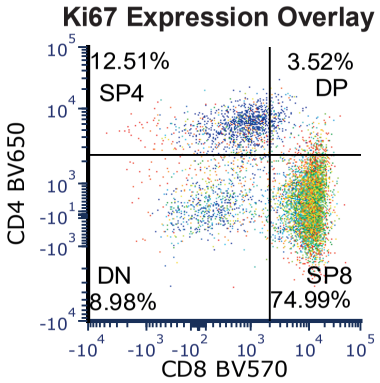

SSC-H (x 1000)
2983
2212.4
1441.8
671.2
-99.4
Cells
59.10%
0
295.2 590.4 885.5
FSC-H (x 1000)

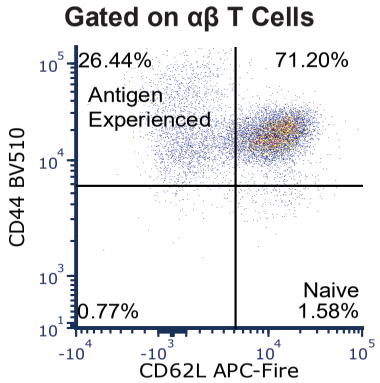

FSC-A (x 1000)
580.8
429.8
278.8
127.8
-23.2
Single Cells
91.62%
0
338.1 676.2 1014.2
FSC-H (x 1000)

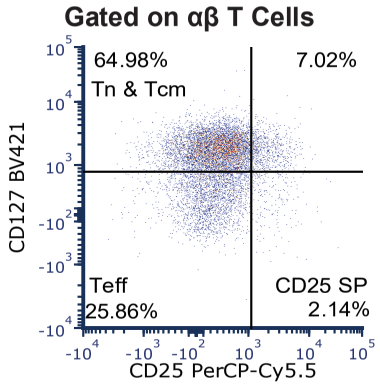

FSC-A (x 1000)
421
315.8
210.5
105.3
0
Live Cells
85.02%
ZombieGreen

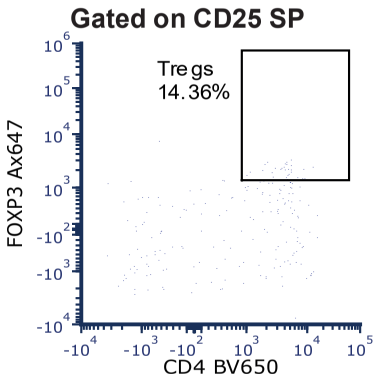

TCRβ PE-Cy7
αβ T Cells
2.09%
CD3 SP
0.75%
CD3ε BV785

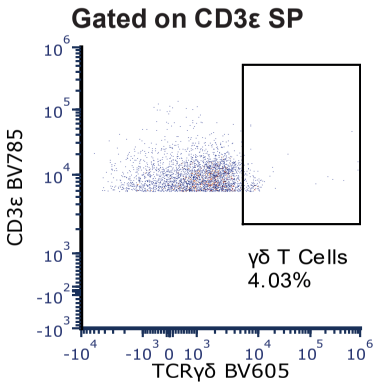

Gated on αβ T Cells
12.05%
SP4
4.21%
DP
DN
8.67%
SP8
75.07%
CD4 BV650
CD8 BV570

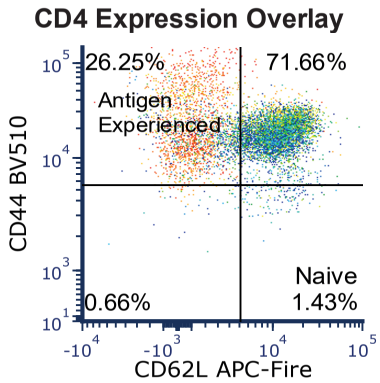

Ki67 Expression Overlay
12.51%
SP4
3.52%
DP
DN
8.98%
SP8
74.99%
CD4 BV650
CD8 BV570

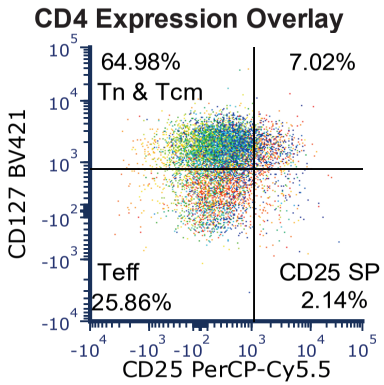

Gated on αβ T Cells
26.44%
71.20%
Antigen
Experienced
0.77%
Naive
1.58%
CD44 BV510
CD62L APC-Fire


Gated on αβ T Cells
64.98%
7.02%
Tn & Tcm
Teff
25.86%
CD25 SP
2.14%
CD127 BV421
CD25 PerCP-Cy5.5


Gated on CD25 SP
Tregs
14.36%
FOXP3 Ax647
CD4 BV650


Gated on CD3ε SP
γδ T Cells
4.03%
CD3ε BV785
TCRγδ BV605


CD4 Expression Overlay
26.25%
71.66%
Antigen
Experienced
0.66%
Naive
1.43%
CD44 BV510
CD62L APC-Fire


CD4 Expression Overlay
64.98%
7.02%
Tn & Tcm
Teff
25.86%
CD25 SP
2.14%
CD127 BV421
CD25 PerCP-Cy5.5

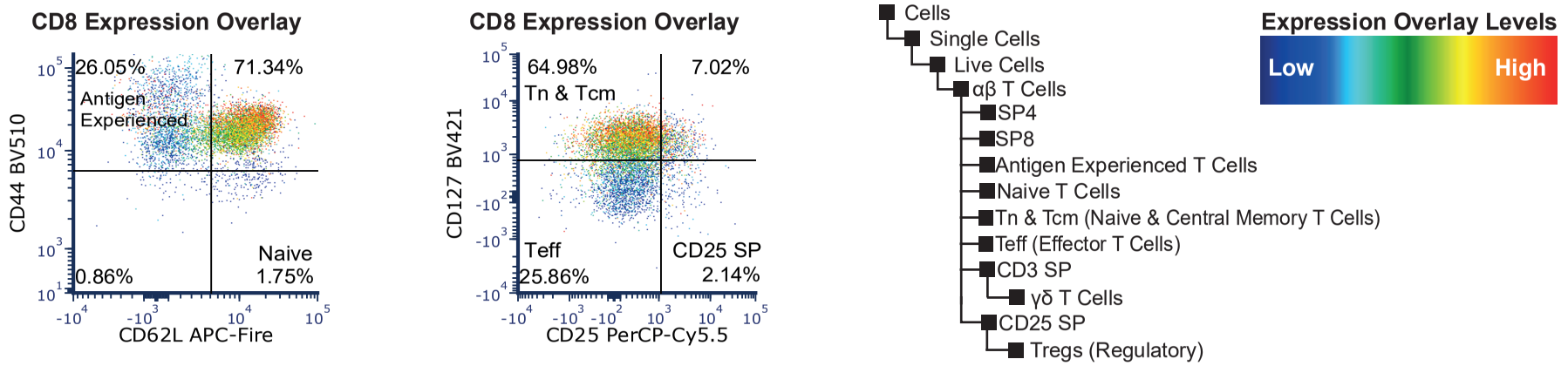
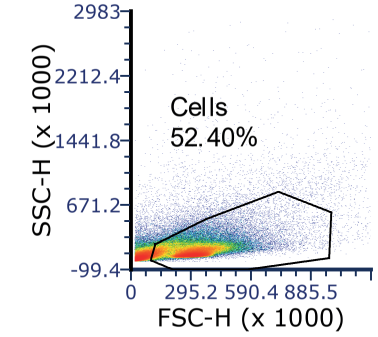
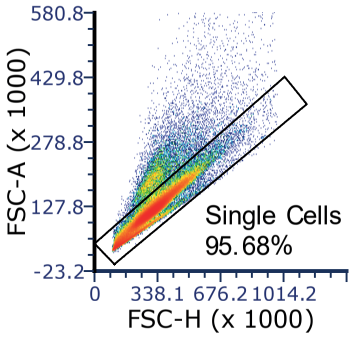
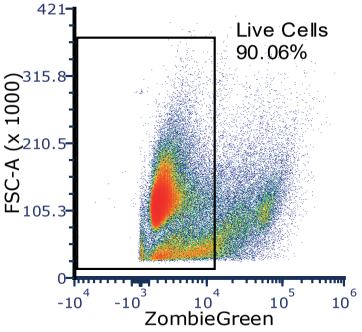
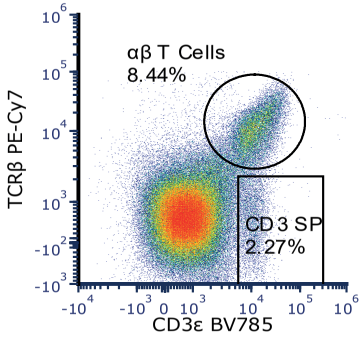


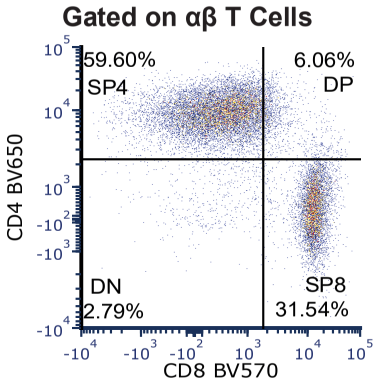
Figure 6.16. Cre MEF-MultiRTOC recipient lymph node T cell analysis: 22 weeks post-transplantation. Representative FACS plots and gating hierarchy for Cre MEF recipient lymph node T cells 22 weeks post transplantation (animal ID 416). Axillary, inguinal and cervical lymph nodes were collected. *Foxn1*$^{G/G}$ transplant recipients received three RTOC each consisting of 600,000 Cre MEFs + 100,000 DN Thymocytes +100,000 WT MEFs.

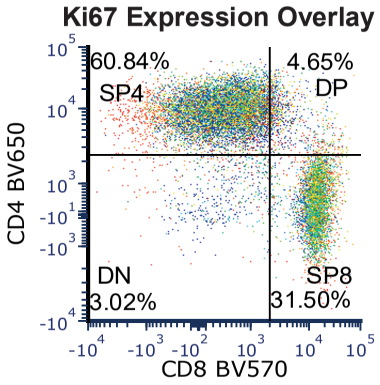

SSC-H (x 1000)
2983
2212.4
1441.8
671.2
-99.4
Cells
52.40%
0
295.2 590.4 885.5
FSC-H (x 1000)

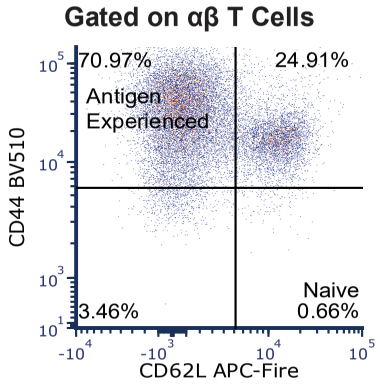

FSC-A (x 1000)
580.8
429.8
278.8
127.8
-23.2
Single Cells
95.68%
0
338.1 676.2 1014.2
FSC-H (x 1000)

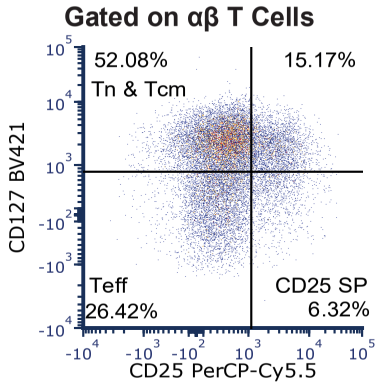

FSC-A (x 1000)
421
315.8
210.5
105.3
0
Live Cells
90.06%
ZombieGreen

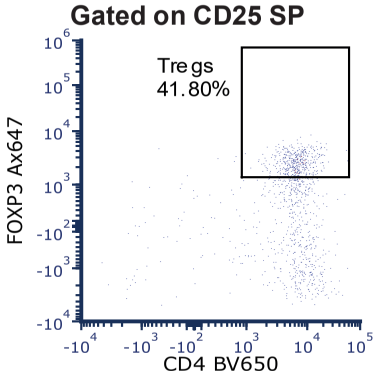

TCRβ PE-Cy7
αβ T Cells
8.44%
CD3 SP
2.27%
CD3ε BV785

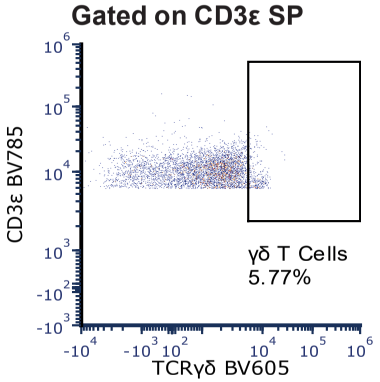

Gated on αβ T Cells
59.60%
SP4
6.06%
DP
DN
2.79%
SP8
31.54%
CD4 BV650
CD8 BV570

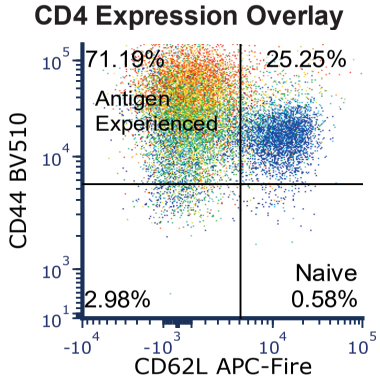

Ki67 Expression Overlay
60.84%
SP4
4.65%
DP
DN
3.02%
SP8
31.50%
CD4 BV650
CD8 BV570

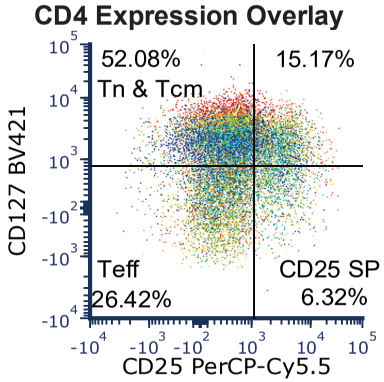

Gated on αβ T Cells
70.97%
Antigen
Experienced
24.91%
3.46%
Naive
0.66%
CD44 BV510
CD62L APC-Fire


Gated on αβ T Cells
52.08%
Tn & Tcm
15.17%
Teff
26.42%
CD25 SP
6.32%
CD127 BV421
CD25 PerCP-Cy5.5


Gated on CD25 SP
Tregs
41.80%
FOXP3 Ax647
CD4 BV650


Gated on CD3ε SP
γδ T Cells
5.77%
CD3ε BV785
TCRγδ BV605


CD4 Expression Overlay
71.19%
Antigen
Experienced
25.25%
2.98%
Naive
0.58%
CD44 BV510
CD62L APC-Fire


CD4 Expression Overlay
52.08%
Tn & Tcm
15.17%
Teff
26.42%
CD25 SP
6.32%
CD127 BV421
CD25 PerCP-Cy5.5

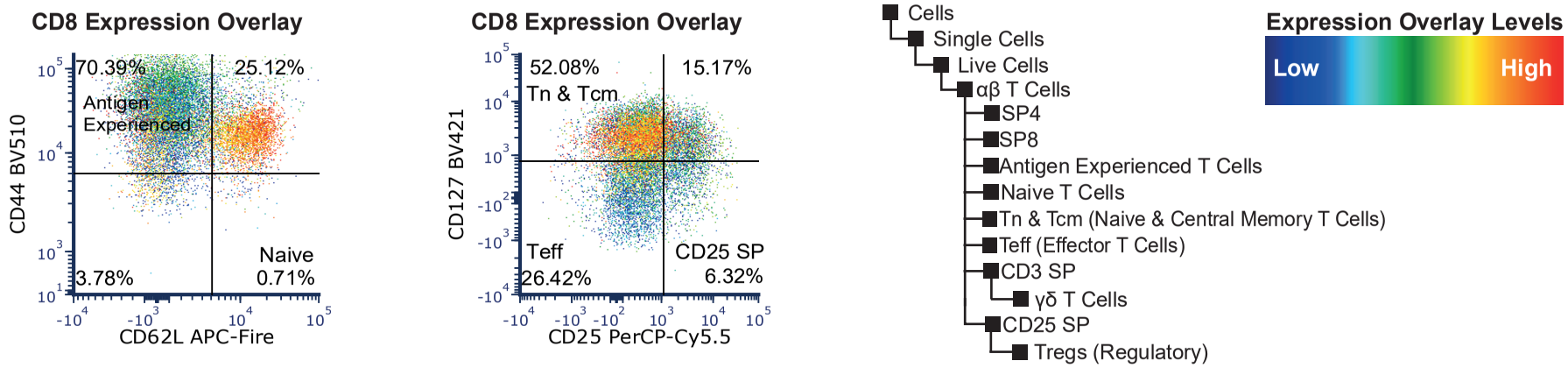
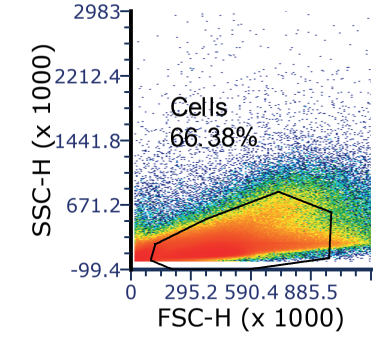
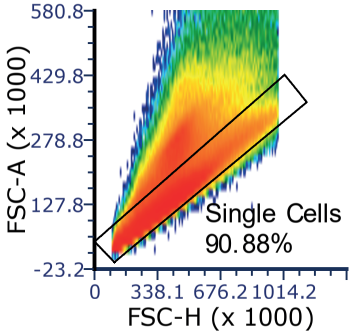
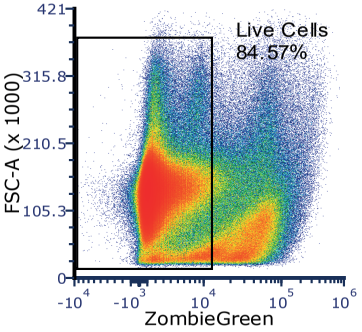
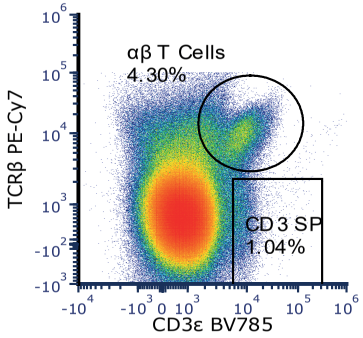


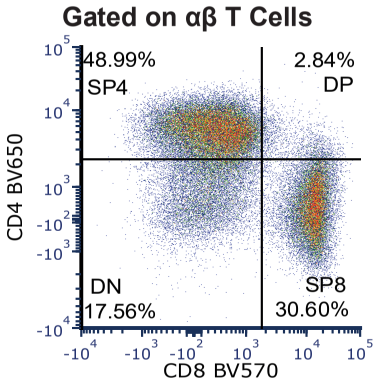
Figure 6.17. iTEC-MultiRTOC recipient lymph node T cell analysis: 22 weeks post-transplantation. Representative FACS plots and gating hierarchy for iTEC recipient lymph node T cells 22 weeks post transplantation (animal ID 495). Axillary, inguinal and cervical lymph nodes were collected. *Foxn1*$^{G/G}$ transplant recipients received three RTOC each consisting of 600,000 iTEC + 100,000 DN Thymocytes +100,000 WT MEFs.

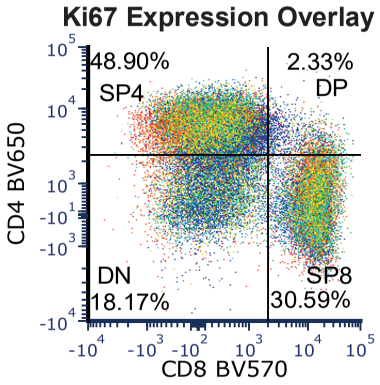

SSC-H (x 1000)
2983
2212.4
1441.8
671.2
-99.4
Cells
66.38%
0
295.2 590.4 885.5
FSC-H (x 1000)

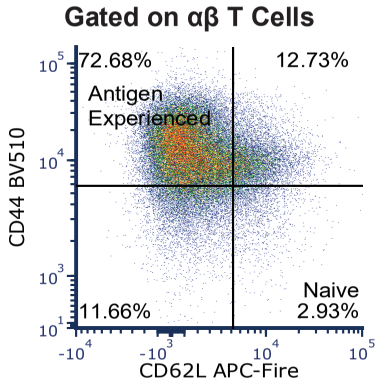

FSC-A (x 1000)
580.8
429.8
278.8
127.8
-23.2
Single Cells
90.88%
0
338.1 676.2 1014.2
FSC-H (x 1000)

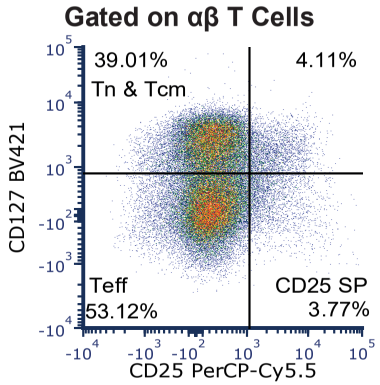

FSC-A (x 1000)
421
315.8
210.5
105.3
0
Live Cells
84.57%
ZombieGreen

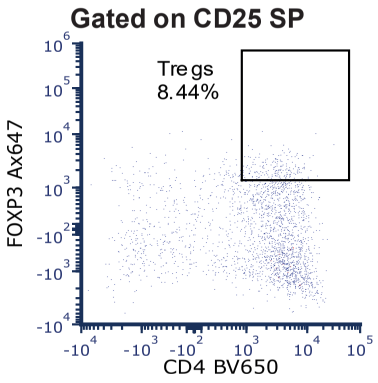

TCRβ PE-Cy7
αβ T Cells
4.30%
CD3 SP
1.04%
CD3ε BV785

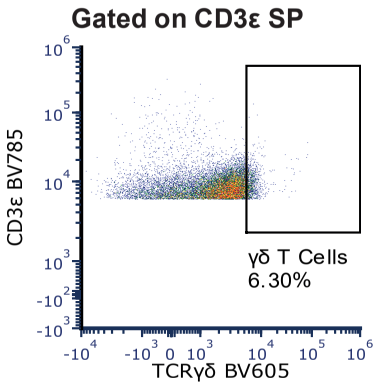

Gated on αβ T Cells
CD4 BV650
48.99%
SP4
2.84%
DP
DN
17.56%
SP8
30.60%
CD8 BV570

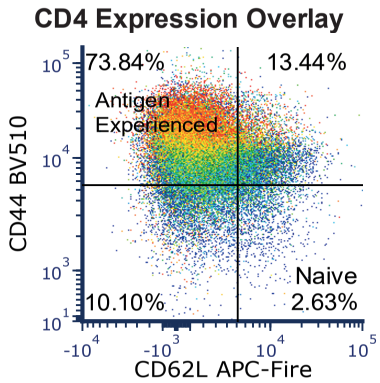

Ki67 Expression Overlay
CD4 BV650
48.90%
SP4
2.33%
DP
DN
18.17%
SP8
30.59%
CD8 BV570

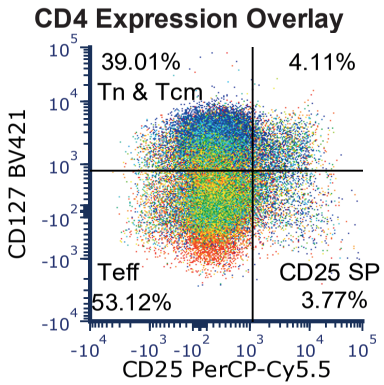

Gated on αβ T Cells
CD44 BV510
72.68%
Antigen
Experienced
12.73%
11.66%
Naive
2.93%
CD62L APC-Fire


Gated on αβ T Cells
CD127 BV421
39.01%
Tn & Tcm
4.11%
Teff
53.12%
CD25 SP
3.77%
CD25 PerCP-Cy5.5


Gated on CD25 SP
FOXP3 Ax647
Tregs
8.44%
CD4 BV650


Gated on CD3ε SP
CD3ε BV785
γδ T Cells
6.30%
TCRγδ BV605


CD4 Expression Overlay
CD44 BV510
73.84%
Antigen
Experienced
13.44%
10.10%
Naive
2.63%
CD62L APC-Fire


CD4 Expression Overlay
CD127 BV421
39.01%
Tn & Tcm
4.11%
Teff
53.12%
CD25 SP
3.77%
CD25 PerCP-Cy5.5

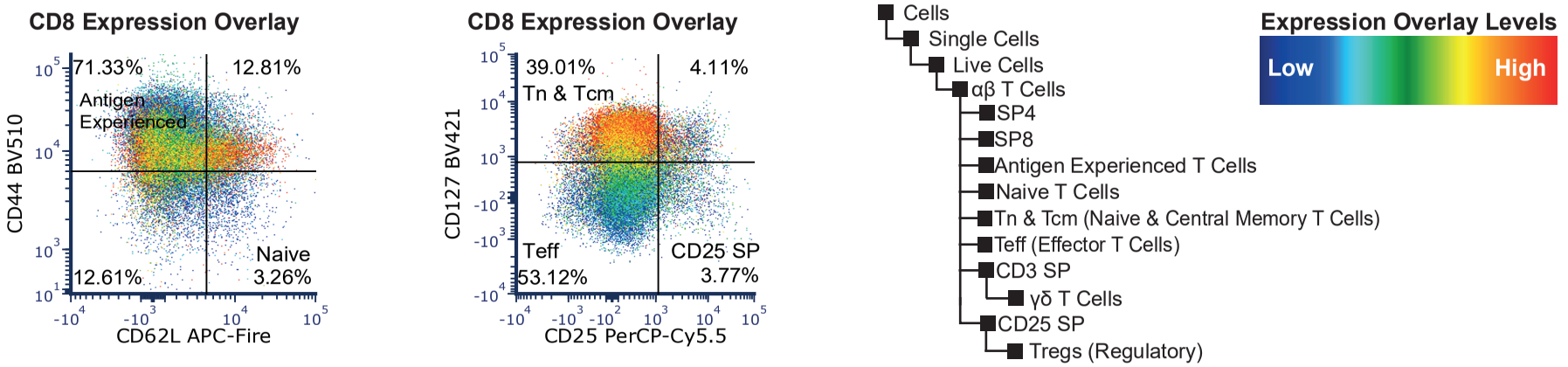
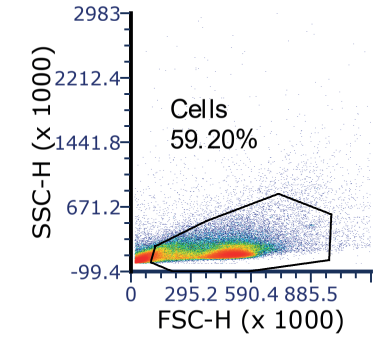
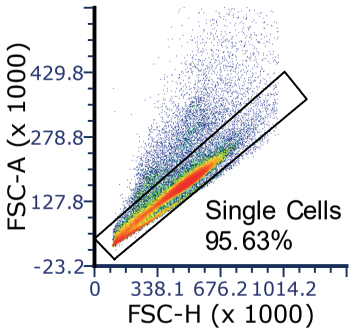
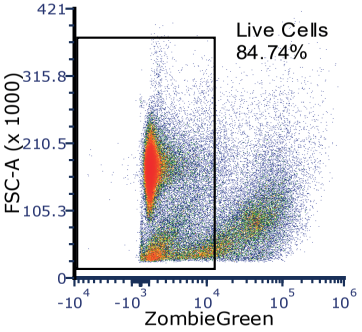
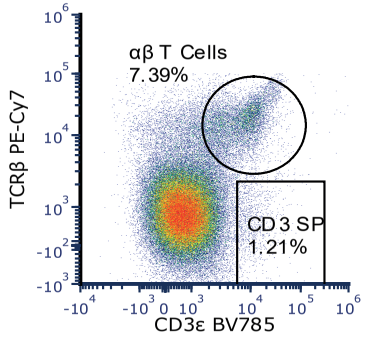


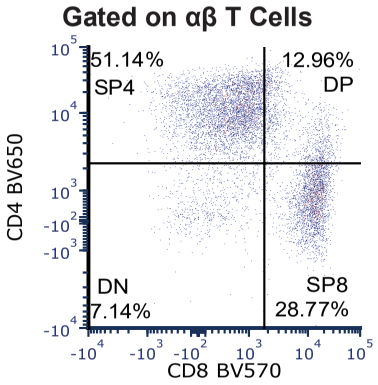
Figure 6.18. Sick iTEC MultiRTOC recipient lymph node T cell analysis: 20 weeks post-transplantation. FACS plots and gating hierarchy for iTEC recipient lymph node T cells 20 weeks post transplantation (animal ID 315). Animal 315 had to be culled prior to experimental endpoint due to rapid weight loss. Axillary, inguinal and cervical lymph nodes were collected. *Foxn1*$^{G/G}$ transplant recipients received three RTOC each consisting of 600,000 iTEC + 100,000 DN Thymocytes +100,000 WT MEFs.

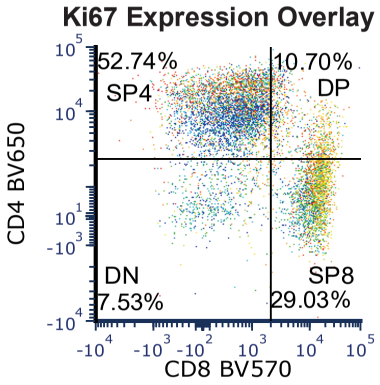

SSC-H (x 1000)
2983
2212.4
1441.8
671.2
-99.4
Cells
59.20%
0
295.2 590.4 885.5
FSC-H (x 1000)

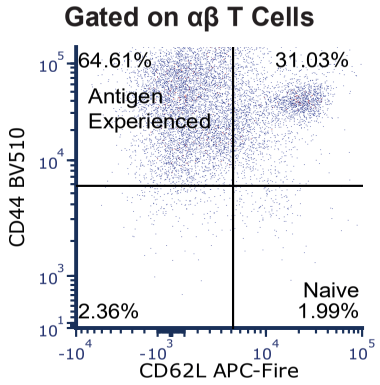

FSC-A (x 1000)
429.8
278.8
127.8
-23.2
Single Cells
95.63%
0
338.1 676.2 1014.2
FSC-H (x 1000)

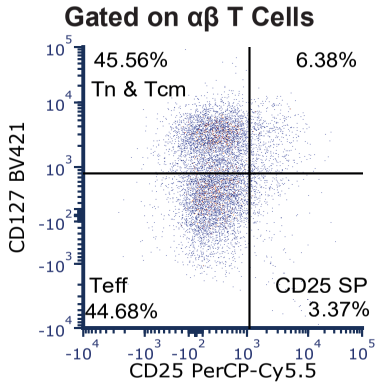

FSC-A (x 1000)
421
315.8
210.5
105.3
0
Live Cells
84.74%
ZombieGreen

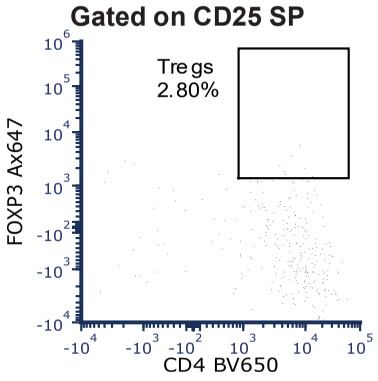

TCRβ PE-Cy7
αβ T Cells
7.39%
CD3 SP
1.21%
CD3ε BV785

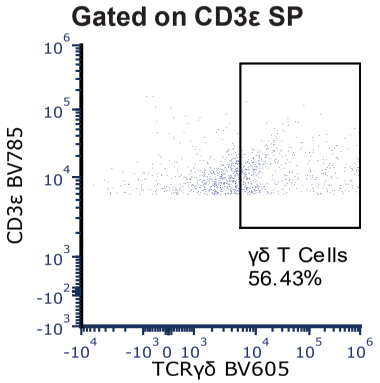

Gated on αβ T Cells
CD4 BV650
51.14%
SP4
12.96%
DP
DN
7.14%
SP8
28.77%
CD8 BV570

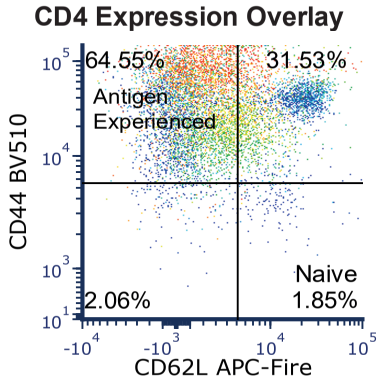

Ki67 Expression Overlay
CD4 BV650
52.74%
SP4
10.70%
DP
DN
7.53%
SP8
29.03%
CD8 BV570

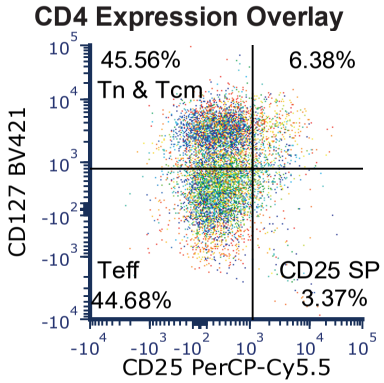

Gated on αβ T Cells
CD44 BV510
64.61%
Antigen
Experienced
31.03%
2.36%
Naive
1.99%
CD62L APC-Fire


Gated on αβ T Cells
CD127 BV421
45.56%
Tn & Tcm
6.38%
Teff
44.68%
CD25 SP
3.37%
CD25 PerCP-Cy5.5


Gated on CD25 SP
FOXP3 Ax647
Tregs
2.80%
CD4 BV650


Gated on CD3ε SP
CD3ε BV785
γδ T Cells
56.43%
TCRγδ BV605


CD4 Expression Overlay
CD44 BV510
64.55%
Antigen
Experienced
31.53%
2.06%
Naive
1.85%
CD62L APC-Fire


CD4 Expression Overlay
CD127 BV421
45.56%
Tn & Tcm
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Teff
44.68%
CD25 SP
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CD25 PerCP-Cy5.5

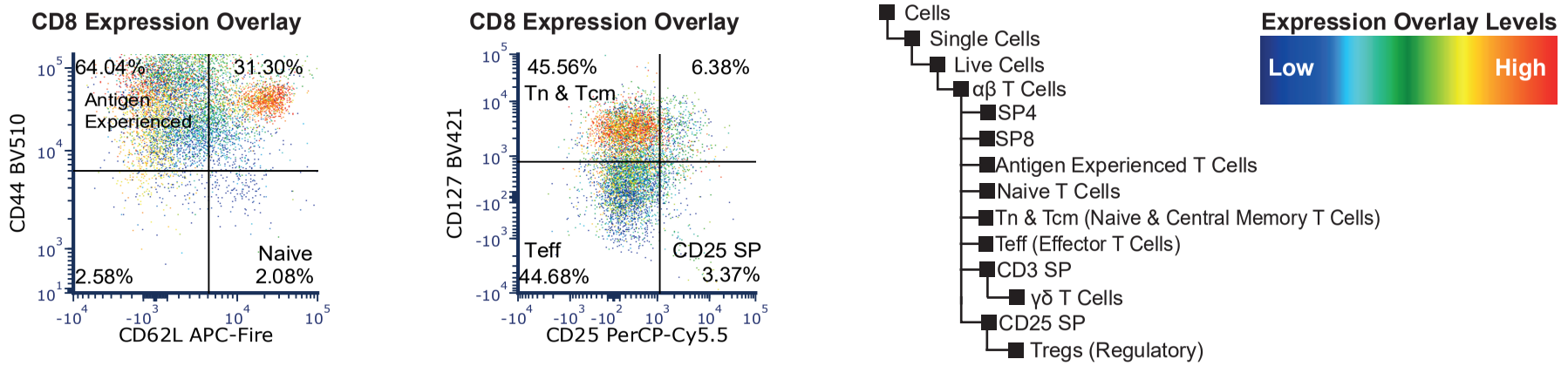


Figure 6.19. Sick iTEC MultiRTOC recipient lymph node T cell analysis: 17 weeks post-transplantation. FACS plots and gating hierarchy for iTEC recipient lymph node T cells 17 weeks post transplantation (animal ID 501). Animal 501 had to be culled prior to experimental endpoint due to rapid weight loss. Axillary, inguinal and cervical lymph nodes were collected. *Foxn1*$^{G/G}$ transplant recipients received three RTOC each consisting of 600,000 iTEC + 100,000 DN Thymocytes +100,000 WT MEFs.

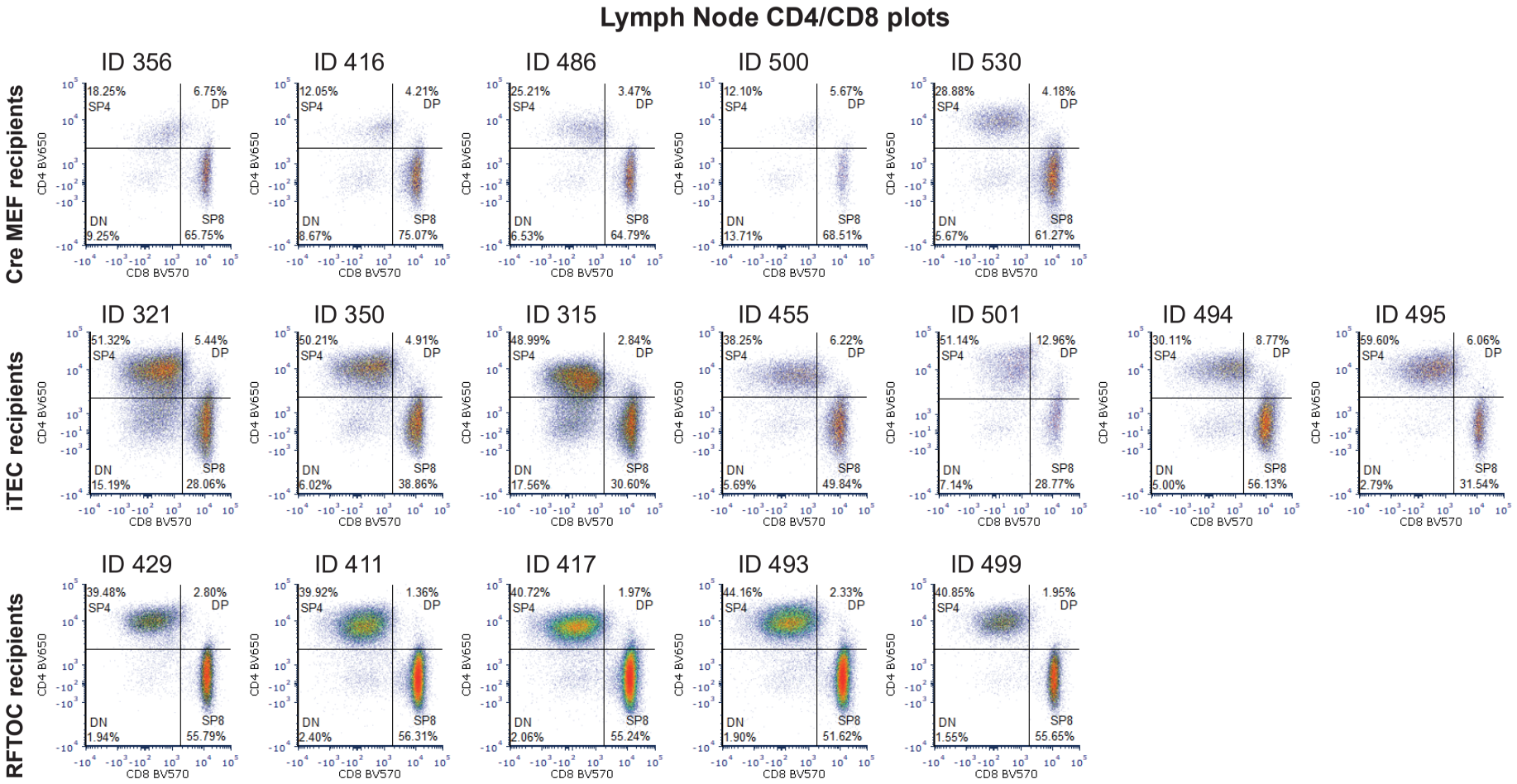
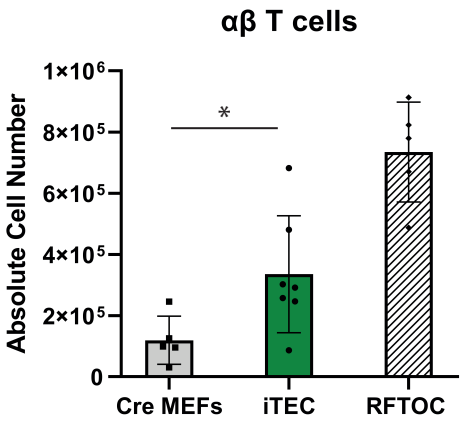


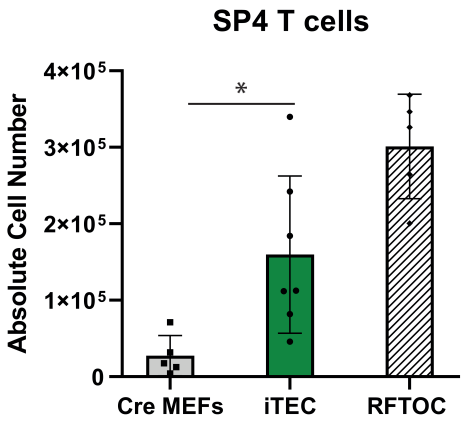
Figure 6.20. Lymph Node CD4/CD8 plots. Collated FACS plots of CD4 and CD8 T cell profles of *Foxn1*$^{G/G}$ recipients at experimental endpoint. Experimental endpoint is 22 weeks for all animals except ID 315 (20 weeks) and 501 (17 weeks). All plots are gated on total αβ T cells (CD3ε^{+}TCRβ^{+}).

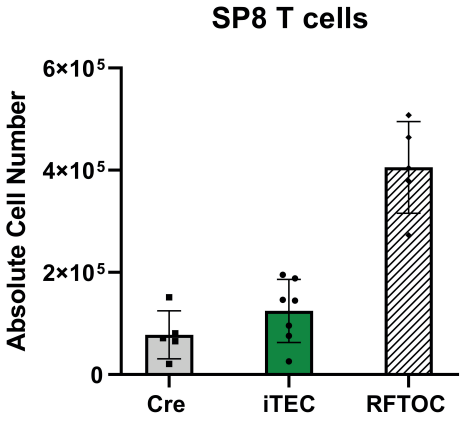
Lymph Node T Cell Analysis											
Condition	**Surgery Rep**	**Animal ID**	**αβ T cells**								**CD3 SP**
			Total	**SP4**	**SP8**	**Regulatory**	**Naïve**	**Naïve & central memory**	**Effector**	**Antigen Experienced**	**γδ T cells**
Cre MEFs	Rep A	**356**	98826	17654	65030	28	1638	67578	28308	29134	30
	Rep B	**416**	95410	12450	71240	290	3280	61390	25270	21330	980
	Rep B	**486**	125622	31780	80514	462	2786	76398	38094	39270	1484
	Rep C	**500**	30352	4284	20706	14	2422	17416	9576	6468	322
	Rep C	**530**	245028	71008	151312	154	8904	95942	114478	132048	980
		Mean	**119048 ± 70355**	**27435 ± 23553**	**77760 ± 42136**	**190 ± 169**	**3806 ± 2605**	**63745 ± 25936**	**43145 ± 36825**	**45650 ± 44508**	**759 ± 519**
iTEC	Rep A	**321**	681960	339865	195321	9558	53950	399721	207411	436096	93
	Rep A	**350**	480326	242270	188342	12866	8428	211386	204540	275030	44
	Rep A	***315**	246084	111744	75960	768	24000	95272	131868	130296	868
	Rep B	**455**	291046	112448	144956	7294	7602	116606	124740	169260	2044
	Rep B	***501**	86130	45700	25940	80	2830	39040	38770	48460	4140
	Rep C	**494**	257430	81730	146280	1290	6380	129690	107330	140310	360
	Rep C	**495**	302138	184083	95655	7945	8278	157063	80203	194810	2695
		Mean	**335016 ± 177431**	**159691 ± 95229**	**124636 ± 57179**	**5686 ± 4616**	**15924 ± 16723**	**164111 ± 107981**	**127837 ± 57151**	**199180 ± 115711**	**1463 ± 1438**
RFTOC	Rep A	**429**	823269	326223	464477	18757	40174	612762	151435	400226	1376
	Rep B	**411**	670520	264630	378660	10470	140250	445210	159190	167120	2570
	Rep B	**417**	912860	368240	507590	25950	224830	592910	214810	228920	2510
	Rep C	**493**	779430	346400	404340	20870	198320	540210	154720	210520	4080
	Rep C	**499**	487375	200340	272615	7753	61460	341950	86328	164570	1925
		Mean	**734691± 146202**	**301167 ± 61112**	**405536 ± 80350**	**16760 ± 6724**	**133007 ± 72783**	**506608 ± 100725**	**153297 ± 40765**	**234271 ± 86604**	**2492 ± 905**

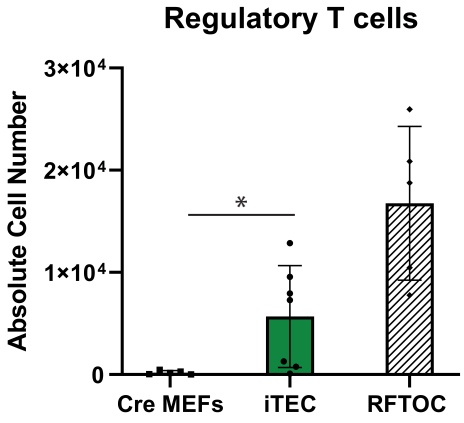
Table 6.5. Absolute cell numbers of lymph node T cells at experimental endpoint. Mean ± SD.

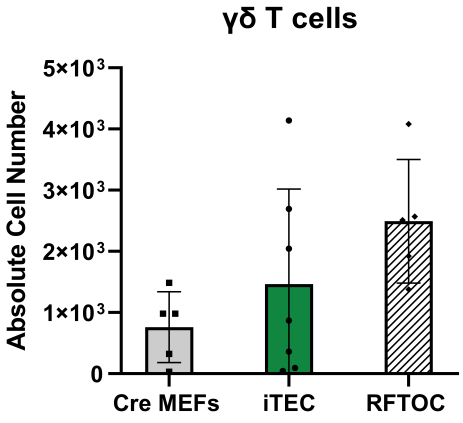
Lymph Node T Cell Analysis

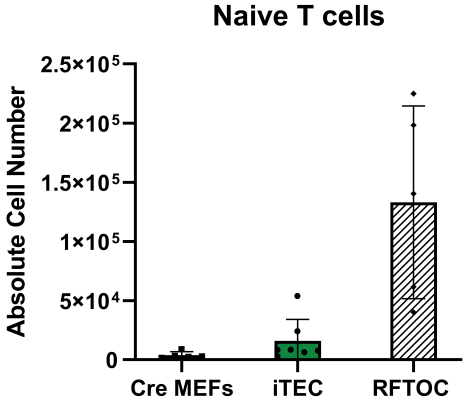












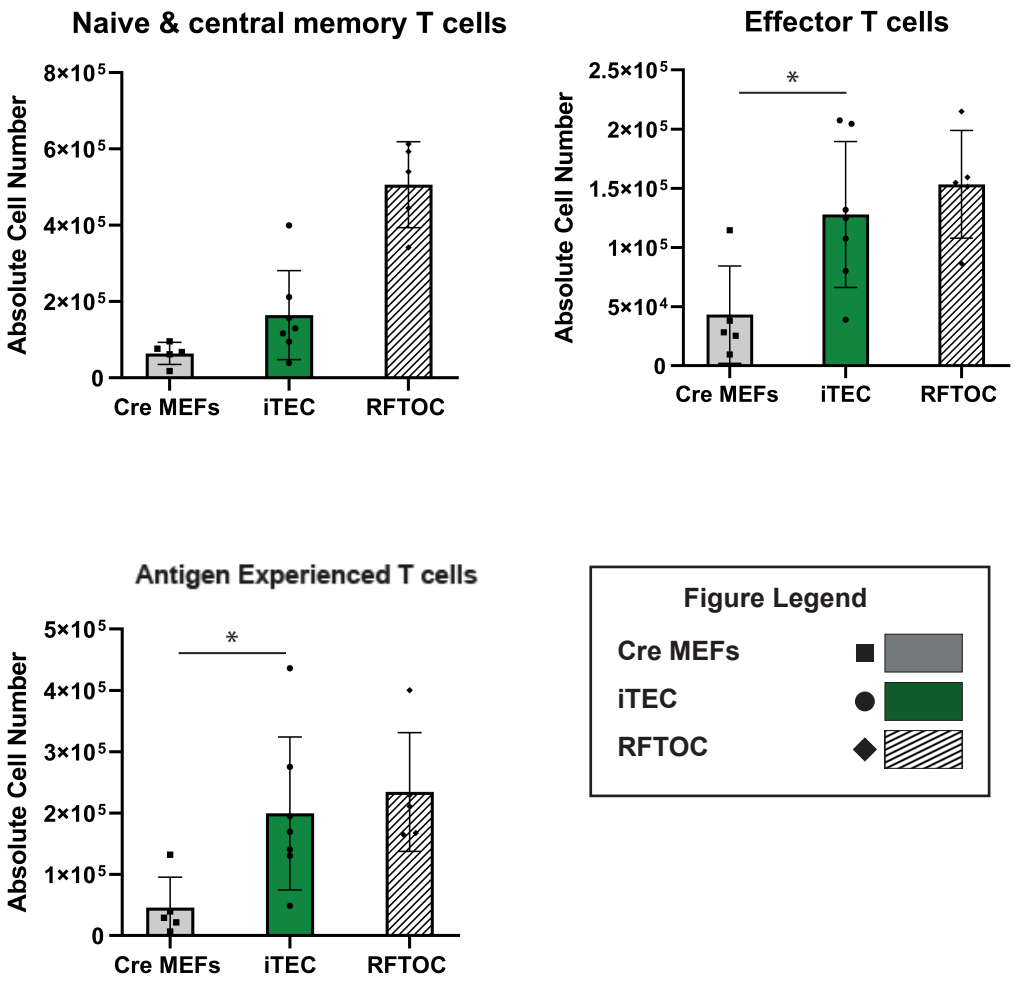


Figure 6.21. Absolute cell numbers of T cells present in lymph nodes of *Foxn1*$^{G/G}$ transplanted mice at experimental endpoint. Each datapoint represents one animal. αβ T cells (CD3ε$^+$TCRβ$^+$), SP4(CD4$^+$), SP8 (CD8$^+$), Regulatory (CD4$^+$FOXP3$^+$), γδ T cells (CD3ε$^+$TCRγδ$^+$), Naive (CD44$^-$CD62L$^+$), Naive & central memory (CD25$^-$CD127$^+$), Effector (CD25$^-$CD127$^-$), Antigen Experienced (CD44$^+$CD62L$^-$). Inguinal, axillary and cervical lymph node T cells were analysed. Absolute cell numbers were normalised depending on number of lymph nodes collected per animal. Graphs display mean±SD. Cre MEFs 3 x (600,000 Cre MEFs + 100,000 WT MEFs + 100,000 DN Thymocytes), iTEC 3 x (600,000 iTEC + 100,000 WT MEFs + 100,000 DN Thymocytes), RFTOC (6 dissociated lobes + 200,000 WT MEFs). Experimental endpoint was 22 weeks post-transplantation with the exception of two animals from the iTEC condition that had to be culled at 20 and 17 weeks due to rapid weight loss and deteriorated health. N=3 independent biological experiments.

Lymph Node T Cell Analysis					
Condition	**Surgery Rep**	**Animal ID**	**SP4**	**SP8**	**SP4:SP8 ratio**
Cre MEFs	Rep A	**356**	17654	65030	0.27
	Rep B	**416**	12450	71240	0.17
	Rep B	**486**	31780	80514	0.39
	Rep C	**500**	4284	20706	0.21
	Rep C	**530**	71008	151312	0.47
		Mean	**27,435**	**77,760**	**0.35**
iTEC	Rep A	**321**	339865	195321	1.74
	Rep A	**350**	242270	188342	1.29
	Rep A	***315**	111744	75960	1.47
	Rep B	**455**	112448	144956	0.78
	Rep B	***501**	45700	25940	1.76
	Rep C	**494**	81730	146280	0.56
	Rep C	**495**	184083	95655	1.92
		Mean	**159,691**	**124,636**	**1.28**
RFTOC	Rep A	**429**	326223	464477	0.70
	Rep B	**411**	264630	378660	0.70
	Rep B	**417**	368240	507590	0.73
	Rep C	**493**	346400	404340	0.86
	Rep C	**499**	200340	272615	0.73
		Mean	**301,167**	**405,536**	**0.74**

Table 6.6. CD4:CD8 ratio of lymph node T cells at experimental endpoint.

6.2.C. Spleen analysis

Representative FACS plots showing the gating strategy used for analysis of splenic T cell populations, and representative examples of each condition, at the experimental endpoints are shown in Figure 6.22 – 6.26; additionally, CD4/CD8 plots are depicted for every recipient and condition (Figure 6.27).

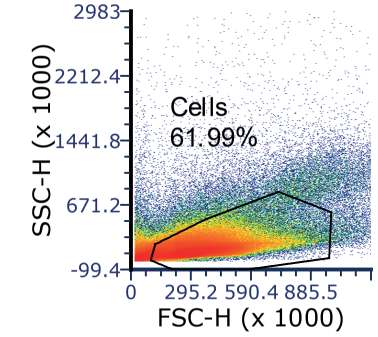
Absolute cell numbers were calculated and adjusted to represent numbers found in the whole spleen of the animal. Absolute cell numbers of the following splenic T cell populations (αβ, SP4, SP8, regulatory, γδ, naïve, naïve and central memory, effector and antigen experienced T cells) at twenty-two weeks post-transplantation are shown in Table 6.7 and Figure 6.28. As mentioned above, two mice (315 and 501) were culled at earlier timepoints (twenty and seventeen weeks) due to rapid weight loss most likely associated with autoimmunity. The two sick mice had the lowest levels of regulatory T cells in the spleen of all the iTEC recipients.

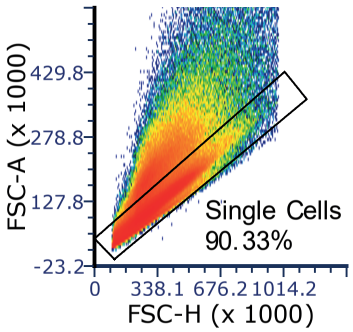
Consistent with the peripheral blood and LN analyses described above, the RFTOC recipients had developed the highest numbers of total T cells, and of all T cell subsets and the iTEC-RTOC recipients had developed higher numbers of all T cell subsets than the Cre MEF recipients. Equal variance (Brown-Forsythe) and normal distribution (Shapiro-Wilk) were tested for all samples as mentioned previously and statistical analysis revealed that the iTEC-RTOC recipients had significantly more effector memory (4.3-fold numerical increase, $p=0.0425$) and antigen experienced (4.9-fold numerical increase, $p=0.0101$) T cells than the negative control Cre MEF recipients. Although not statistically significant, the iTEC-RTOC recipients displayed a 3.3-fold numerical increase in αβ T cells, a 10-fold numerical increase in SP4 T cells and a 7.9-fold numerical increase in regulatory T cells versus negative control Cre MEF recipients.

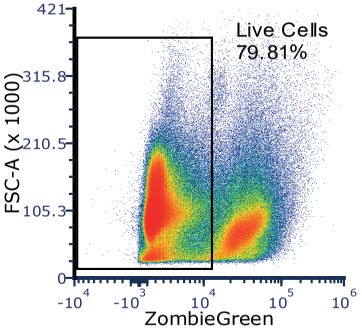
Analysis using expression overlays showed that the T cells from RFTOC-, Cre MEF- and iTEC-RTOC recipients all had similar proliferative profiles, with Ki67 expression being highest in SP4s and intermediate in SP8s. Additionally, SP4 cells were mostly found to be effector memory and antigen experienced T cells, while SP8s were mainly naïve and central memory subsets. No noticeable differences were observed between the healthy cohort and the mice that became sick during the course of the experiment.

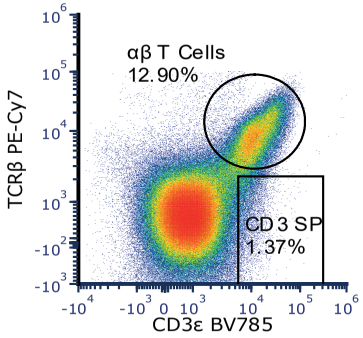
Table 6.8 depicts CD4:CD8 T cell ratios in the spleen for each animal of each experimental condition. Cre MEF recipients averaged at 0.22, iTEC recipients at

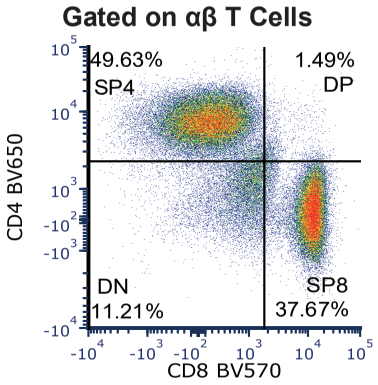
1.84 and RFTOC recipients at 1.30. The considerably lower values of Cre MEF recipient CD4:CD8 ratios (as in the lymph nodes) in comparison to the positive control recipients, again highlights the still present immunodeficient status of the animals. In contrast, similar to the positive controls, five out of seven iTEC recipients display values >1, suggesting immunodeficiency reversal. Although iTEC recipients 321 and 494 display values <1 (0.62 and 0.73), these are still higher than any of the negative control recipient values, still indicative of immunodeficiency reversal.

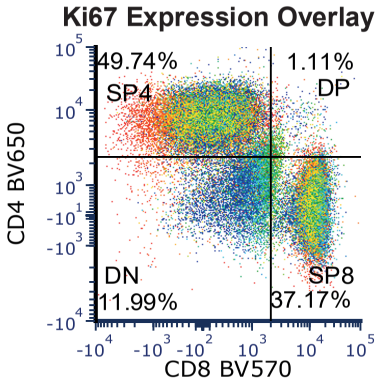

SSC-H (x 1000)
2983
2212.4
1441.8
671.2
-99.4
Cells
61.99%
0
295.2 590.4 885.5
FSC-H (x 1000)

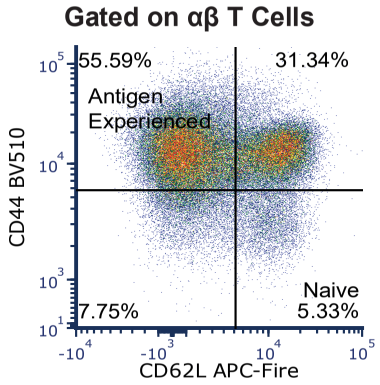

FSC-A (x 1000)
429.8
278.8
127.8
-23.2
Single Cells
90.33%
0
338.1 676.2 1014.2
FSC-H (x 1000)

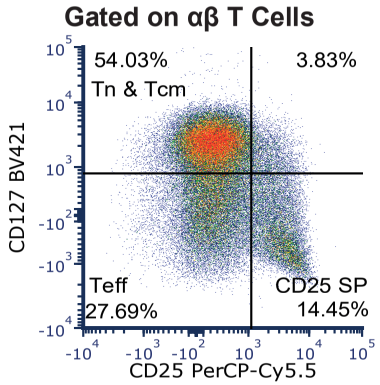

FSC-A (x 1000)
421
315.8
210.5
105.3
0
Live Cells
79.81%
ZombieGreen

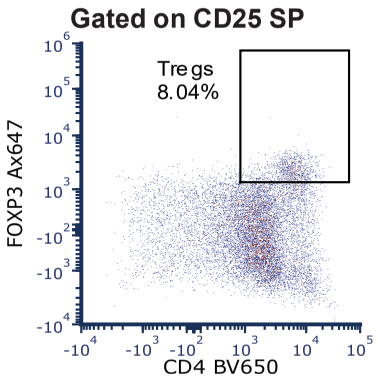

TCRβ PE-Cy7
αβ T Cells
12.90%
CD3 SP
1.37%
CD3ε BV785

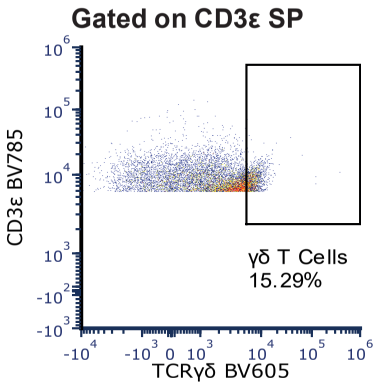

Gated on αβ T Cells
CD4 BV650
49.63%
SP4
1.49%
DP
DN
11.21%
SP8
37.67%
CD8 BV570

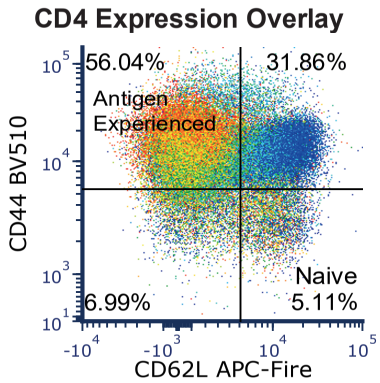

Ki67 Expression Overlay
CD4 BV650
49.74%
SP4
1.11%
DP
DN
11.99%
SP8
37.17%
CD8 BV570

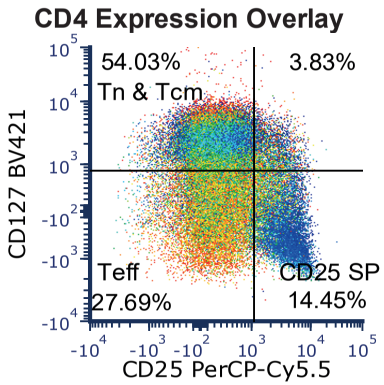

Gated on αβ T Cells
CD44 BV510
55.59%
Antigen
Experienced
31.34%
7.75%
Naive
5.33%
CD62L APC-Fire


Gated on αβ T Cells
CD127 BV421
54.03%
Tn & Tcm
3.83%
Teff
27.69%
CD25 SP
14.45%
CD25 PerCP-Cy5.5


Gated on CD25 SP
FOXP3 Ax647
Tregs
8.04%
CD4 BV650


Gated on CD3ε SP
CD3ε BV785
γδ T Cells
15.29%
TCRγδ BV605


CD4 Expression Overlay
CD44 BV510
56.04%
Antigen
Experienced
31.86%
6.99%
Naive
5.11%
CD62L APC-Fire


CD4 Expression Overlay
CD127 BV421
54.03%
Tn & Tcm
3.83%
Teff
27.69%
CD25 SP
14.45%
CD25 PerCP-Cy5.5

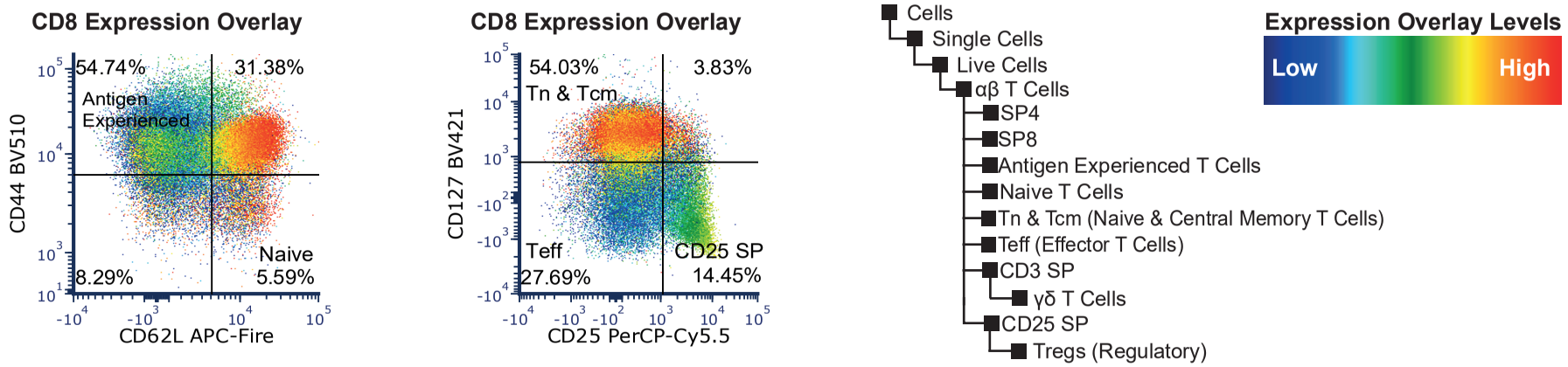
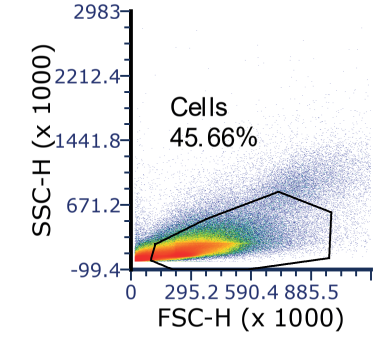


Figure 6.22. RFTOC recipient spleen T cell analysis: 22 weeks post-transplantation. Representative FACS plots and gating hierarchy for RFTOC recipient spleen T cells 22 weeks post transplantation (animal ID 499). *Foxn1*$^{G/G}$ transplant recipients received one RFTOC each consisting of 6 dissociated whole E15.5 thymic lobes + 200,000 WT MEFs.


Cells
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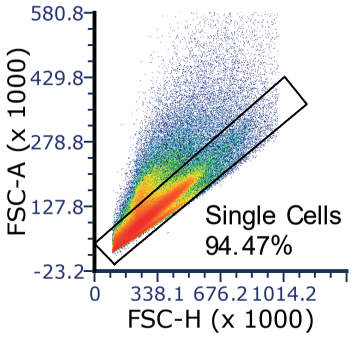
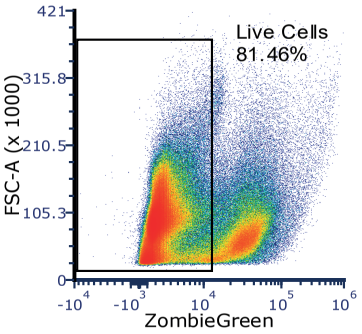
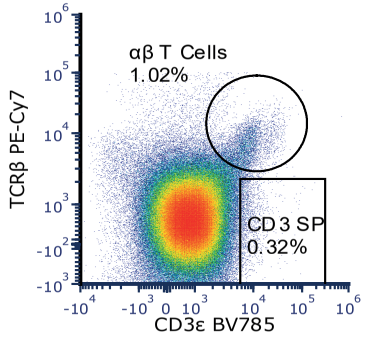
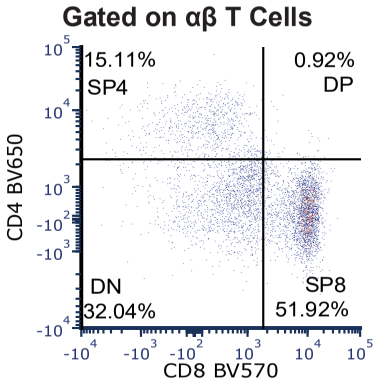
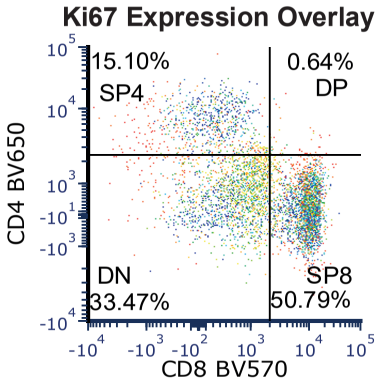
SSC-H (x 1000)
FSC-H (x 1000)

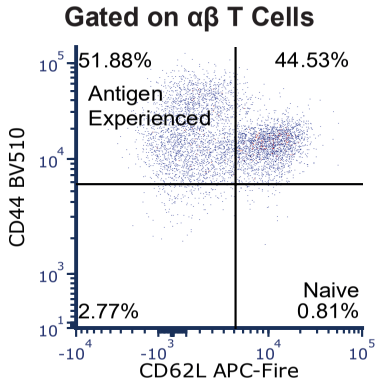

Single Cells
94.47%
FSC-A (x 1000)
FSC-H (x 1000)

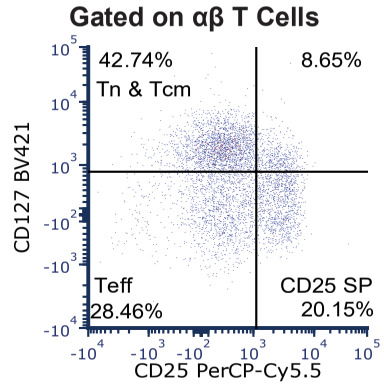

Live Cells
81.46%
FSC-A (x 1000)
ZombieGreen

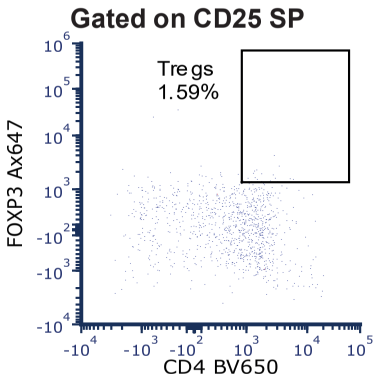

αβ T Cells
1.02%
CD3 SP
0.32%
TCRβ PE-Cy7
CD3ε BV785

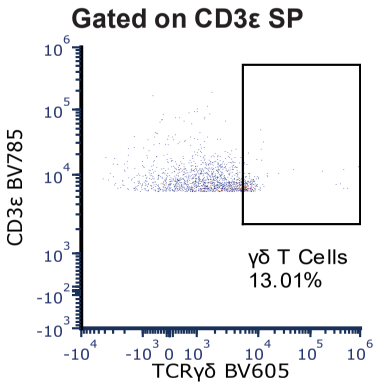

Gated on αβ T Cells
15.11%
SP4
0.92%
DP
DN
32.04%
SP8
51.92%
CD4 BV650
CD8 BV570

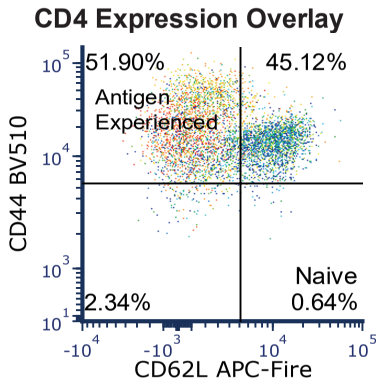

Ki67 Expression Overlay
15.10%
SP4
0.64%
DP
DN
33.47%
SP8
50.79%
CD4 BV650
CD8 BV570

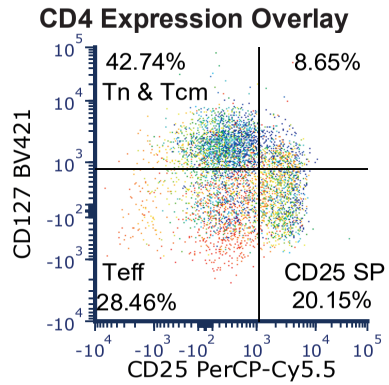

Gated on αβ T Cells
51.88%
44.53%
Antigen
Experienced
2.77%
Naive
0.81%
CD44 BV510
CD62L APC-Fire


Gated on αβ T Cells
42.74%
8.65%
Tn & Tcm
Teff
28.46%
CD25 SP
20.15%
CD127 BV421
CD25 PerCP-Cy5.5


Gated on CD25 SP
Tregs
1.59%
FOXP3 Ax647
CD4 BV650


Gated on CD3ε SP
γδ T Cells
13.01%
CD3ε BV785
TCRγδ BV605


CD4 Expression Overlay
51.90%
45.12%
Antigen
Experienced
2.34%
Naive
0.64%
CD44 BV510
CD62L APC-Fire


CD4 Expression Overlay
42.74%
8.65%
Tn & Tcm
Teff
28.46%
CD25 SP
20.15%
CD127 BV421
CD25 PerCP-Cy5.5

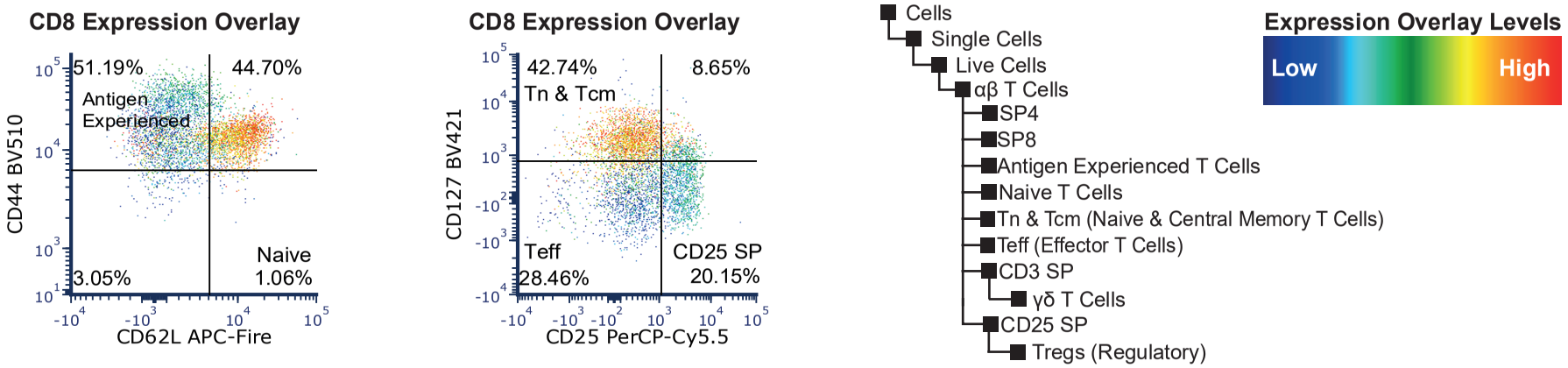
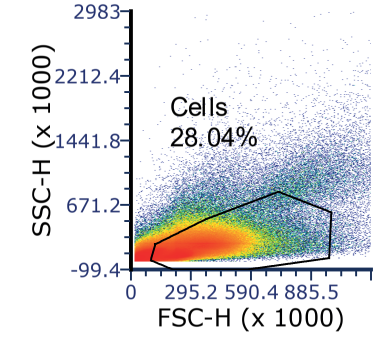
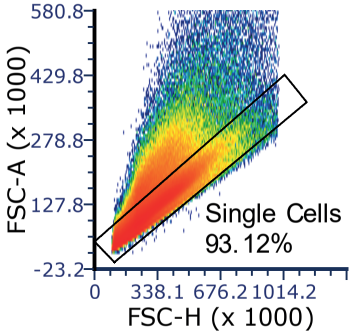
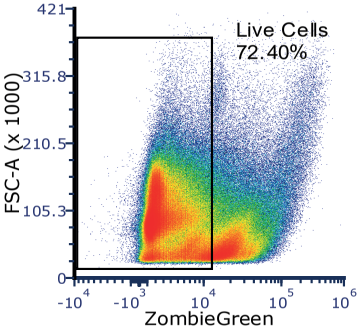
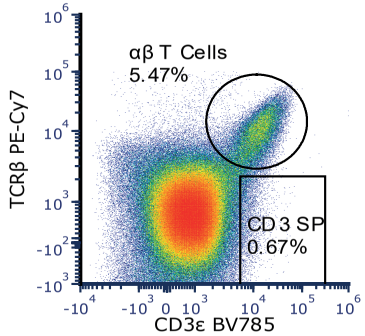


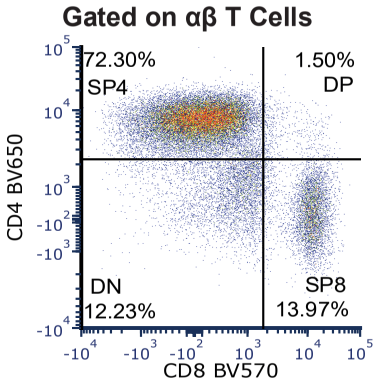
Figure 6.23. Cre MEF-MultiRTOC recipient spleen T cell analysis: 22 weeks post-transplantation. Representative FACS plots and gating hierarchy for Cre MEF recipient spleen T cells 22 weeks post transplantation (animal ID 416). *Foxn1*$^{G/G}$ transplant recipients received three RTOC each consisting of 600,000 Cre MEFs + 100,000 DN Thymocytes +100,000 WT MEFs.

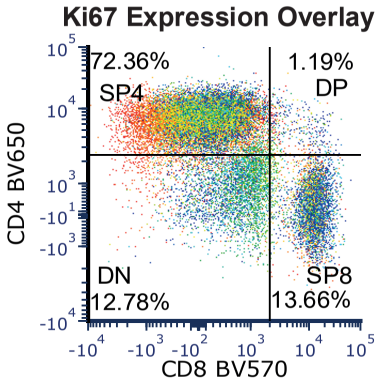

Cells
28.04%
SSC-H (x 1000)
FSC-H (x 1000)

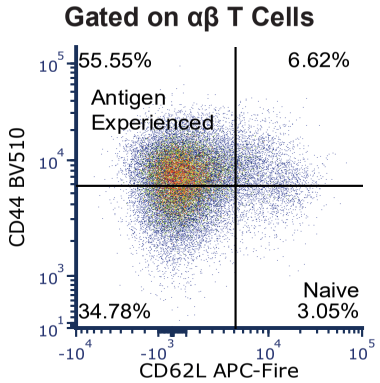

Single Cells
93.12%
FSC-A (x 1000)
FSC-H (x 1000)

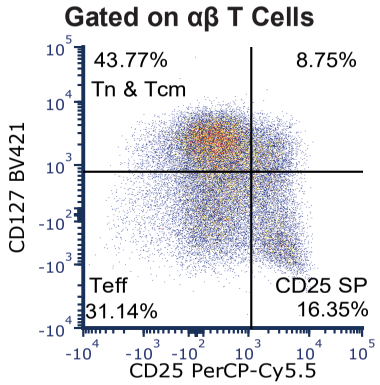

Live Cells
72.40%
FSC-A (x 1000)
ZombieGreen

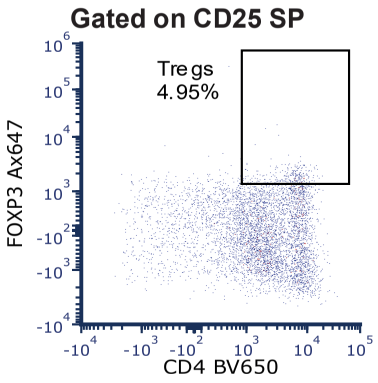

αβ T Cells
5.47%
CD3 SP
0.67%
TCRβ PE-Cy7
CD3ε BV785

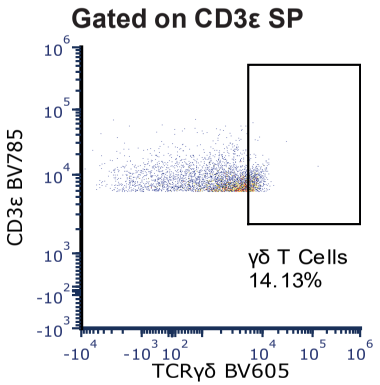

Gated on αβ T Cells
72.30%
SP4
1.50%
DP
DN
12.23%
SP8
13.97%
CD4 BV650
CD8 BV570

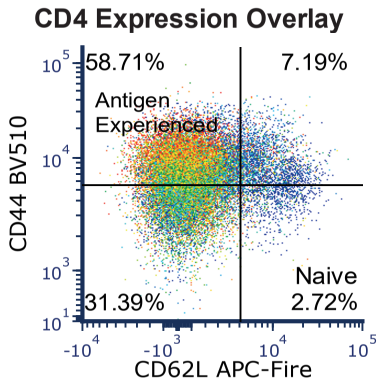

Ki67 Expression Overlay
72.36%
SP4
1.19%
DP
DN
12.78%
SP8
13.66%
CD4 BV650
CD8 BV570

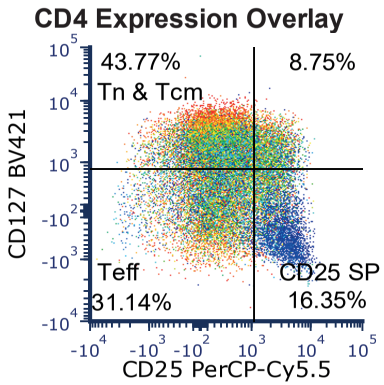

Gated on αβ T Cells
55.55%
6.62%
Antigen
Experienced
34.78%
Naive
3.05%
CD44 BV510
CD62L APC-Fire


Gated on αβ T Cells
43.77%
8.75%
Tn & Tcm
Teff
31.14%
CD25 SP
16.35%
CD127 BV421
CD25 PerCP-Cy5.5


Gated on CD25 SP
Tregs
4.95%
FOXP3 Ax647
CD4 BV650


Gated on CD3ε SP
γδ T Cells
14.13%
CD3ε BV785
TCRγδ BV605


CD4 Expression Overlay
58.71%
7.19%
Antigen
Experienced
31.39%
Naive
2.72%
CD44 BV510
CD62L APC-Fire


CD4 Expression Overlay
43.77%
8.75%
Tn & Tcm
Teff
31.14%
CD25 SP
16.35%
CD127 BV421
CD25 PerCP-Cy5.5

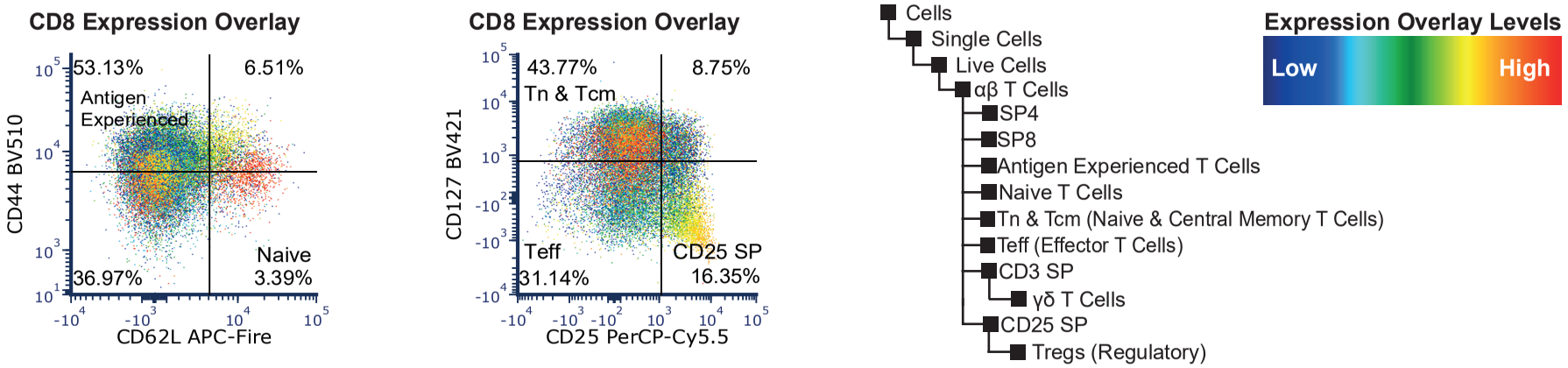
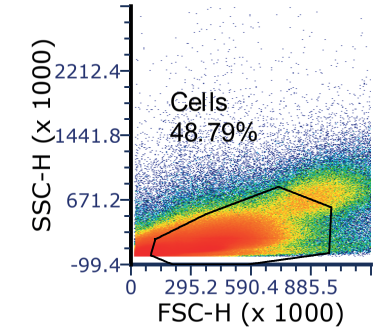
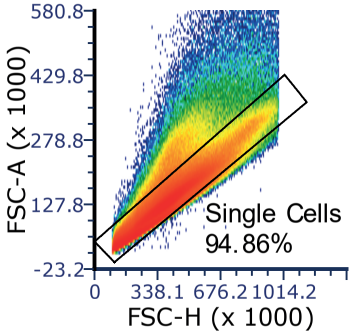
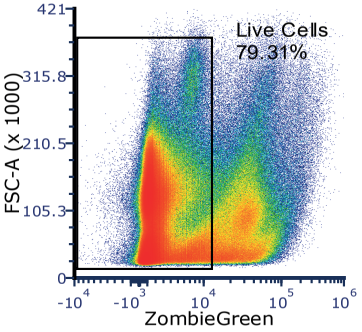
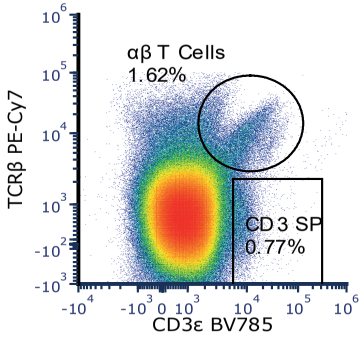


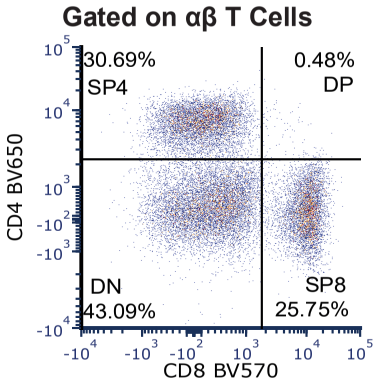
Figure 6.24. iTEC-MultiRTOC recipient spleen T cell analysis: 22 weeks post-transplantation. Representative FACS plots and gating hierarchy for iTEC recipient spleen T cells 22 weeks post transplantation (animal ID 495). *Foxn1*$^{G/G}$ transplant recipients received three RTOC each consisting of 600,000 iTEC + 100,000 DN Thymocytes +100,000 WT MEFs.

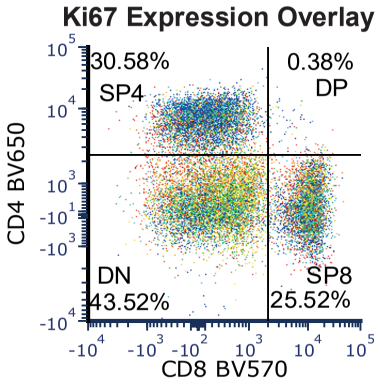

Cells
48.79%
SSC-H (x 1000)
2212.4
1441.8
671.2
-99.4
0
295.2 590.4 885.5
FSC-H (x 1000)

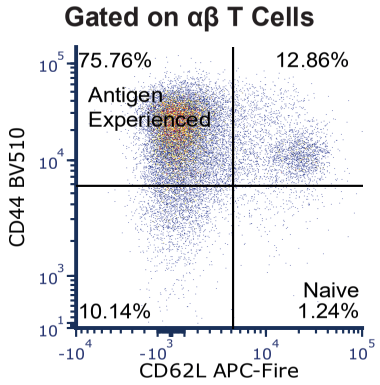

Single Cells
94.86%
FSC-A (x 1000)
580.8
429.8
278.8
127.8
-23.2
0
338.1 676.2 1014.2
FSC-H (x 1000)

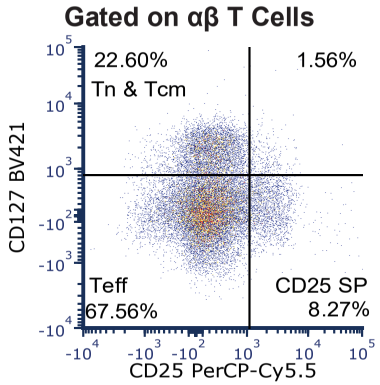

Live Cells
79.31%
FSC-A (x 1000)
421
315.8
210.5
105.3
0
ZombieGreen

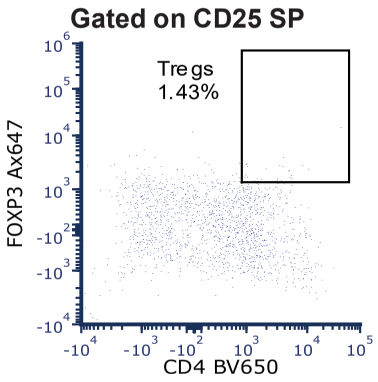

αβ T Cells
1.62%
CD3 SP
0.77%
TCRβ PE-Cy7
CD3ε BV785

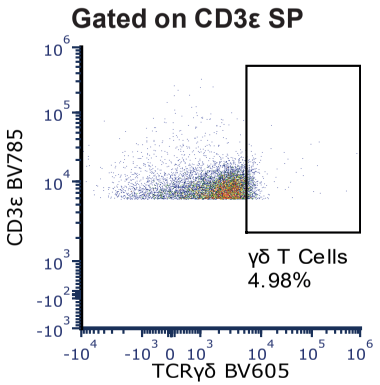

Gated on αβ T Cells
30.69%
SP4
0.48%
DP
DN
43.09%
SP8
25.75%
CD4 BV650
CD8 BV570

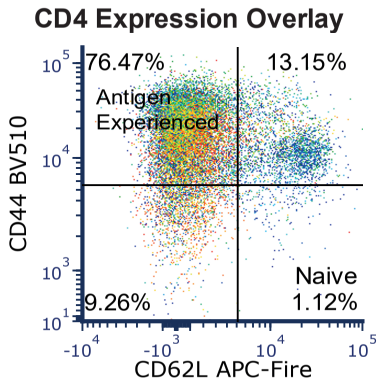

Ki67 Expression Overlay
30.58%
SP4
0.38%
DP
DN
43.52%
SP8
25.52%
CD4 BV650
CD8 BV570

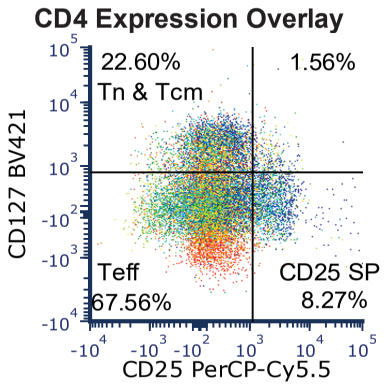

Gated on αβ T Cells
75.76%
12.86%
Antigen
Experienced
10.14%
Naive
1.24%
CD44 BV510
CD62L APC-Fire


Gated on αβ T Cells
22.60%
1.56%
Tn & Tcm
Teff
67.56%
CD25 SP
8.27%
CD127 BV421
CD25 PerCP-Cy5.5


Gated on CD25 SP
Tregs
1.43%
FOXP3 Ax647
CD4 BV650


Gated on CD3ε SP
γδ T Cells
4.98%
CD3ε BV785
TCRγδ BV605


CD4 Expression Overlay
76.47%
13.15%
Antigen
Experienced
9.26%
Naive
1.12%
CD44 BV510
CD62L APC-Fire


CD4 Expression Overlay
22.60%
1.56%
Tn & Tcm
Teff
67.56%
CD25 SP
8.27%
CD127 BV421
CD25 PerCP-Cy5.5

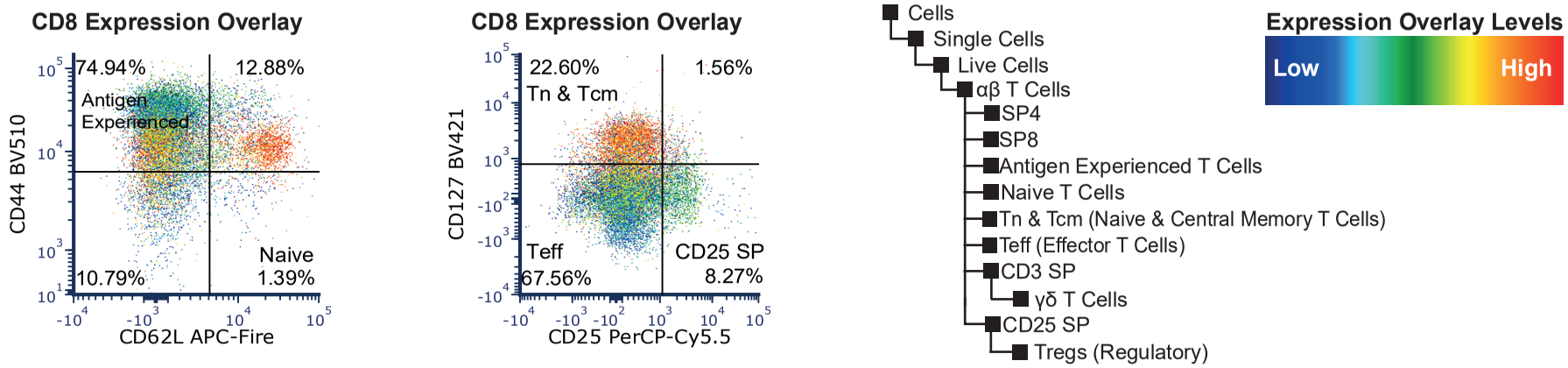
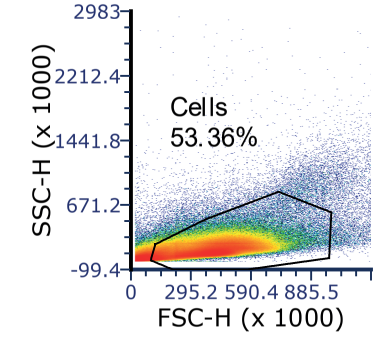
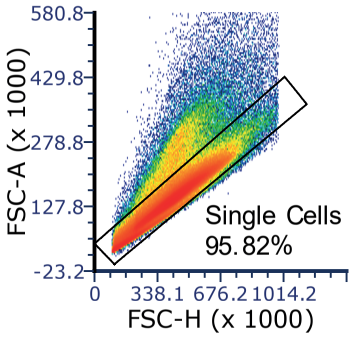
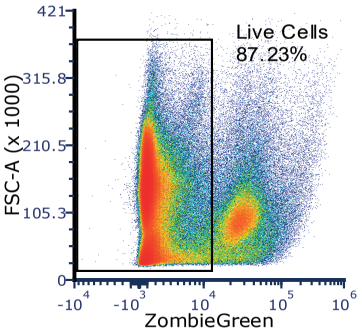
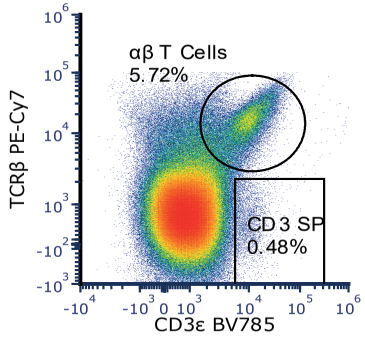


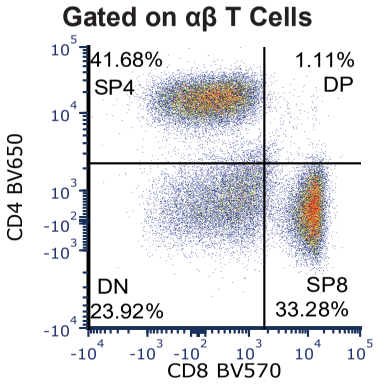
Figure 6.25. Sick iTEC MultiRTOC recipient spleen T cell analysis: 20 weeks post-transplantation. FACS plots and gating hierarchy for iTEC recipient spleen T cells 20 weeks post transplantation (animal ID 315). Animal 315 had to be culled prior to experimental endpoint due to rapid weight loss. *Foxn1*$^{G/G}$ transplant recipients received three RTOC each consisting of 600,000 iTEC + 100,000 DN Thymocytes +100,000 WT MEFs.

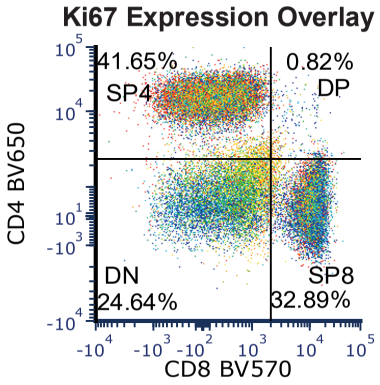

SSC-H (x 1000)
2983
2212.4
1441.8
671.2
-99.4
Cells
53.36%
0
295.2 590.4 885.5
FSC-H (x 1000)

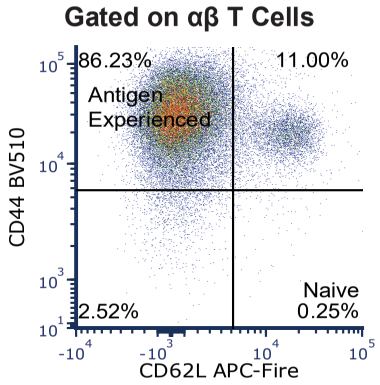

FSC-A (x 1000)
580.8
429.8
278.8
127.8
-23.2
Single Cells
95.82%
0
338.1 676.2 1014.2
FSC-H (x 1000)

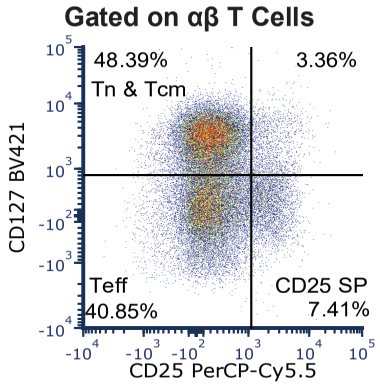

FSC-A (x 1000)
421
315.8
210.5
105.3
0
Live Cells
87.23%
ZombieGreen

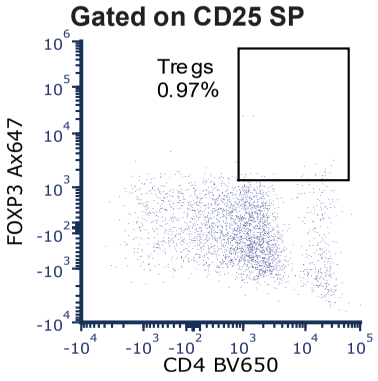

TCRβ PE-Cy7
αβ T Cells
5.72%
CD3 SP
0.48%
CD3ε BV785

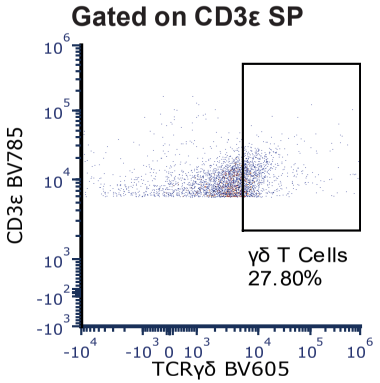

Gated on αβ T Cells
CD4 BV650
41.68%
SP4
1.11%
DP
DN
23.92%
SP8
33.28%
CD8 BV570

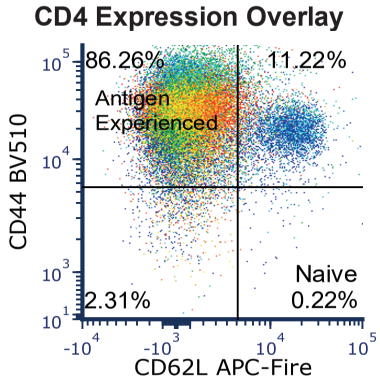

Ki67 Expression Overlay
CD4 BV650
41.65%
SP4
0.82%
DP
DN
24.64%
SP8
32.89%
CD8 BV570

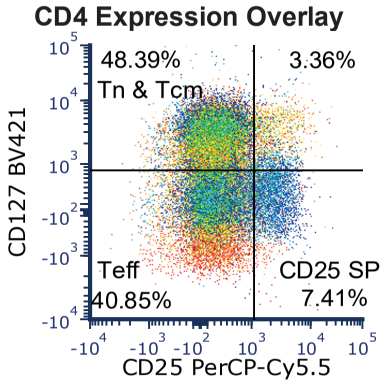

Gated on αβ T Cells
CD44 BV510
86.23%
Antigen
Experienced
11.00%
2.52%
Naive
0.25%
CD62L APC-Fire


Gated on αβ T Cells
CD127 BV421
48.39%
Tn & Tcm
3.36%
Teff
40.85%
CD25 SP
7.41%
CD25 PerCP-Cy5.5


Gated on CD25 SP
FOXP3 Ax647
Tregs
0.97%
CD4 BV650


Gated on CD3ε SP
CD3ε BV785
γδ T Cells
27.80%
TCRγδ BV605


CD4 Expression Overlay
CD44 BV510
86.26%
Antigen
Experienced
11.22%
2.31%
Naive
0.22%
CD62L APC-Fire


CD4 Expression Overlay
CD127 BV421
48.39%
Tn & Tcm
3.36%
Teff
40.85%
CD25 SP
7.41%
CD25 PerCP-Cy5.5

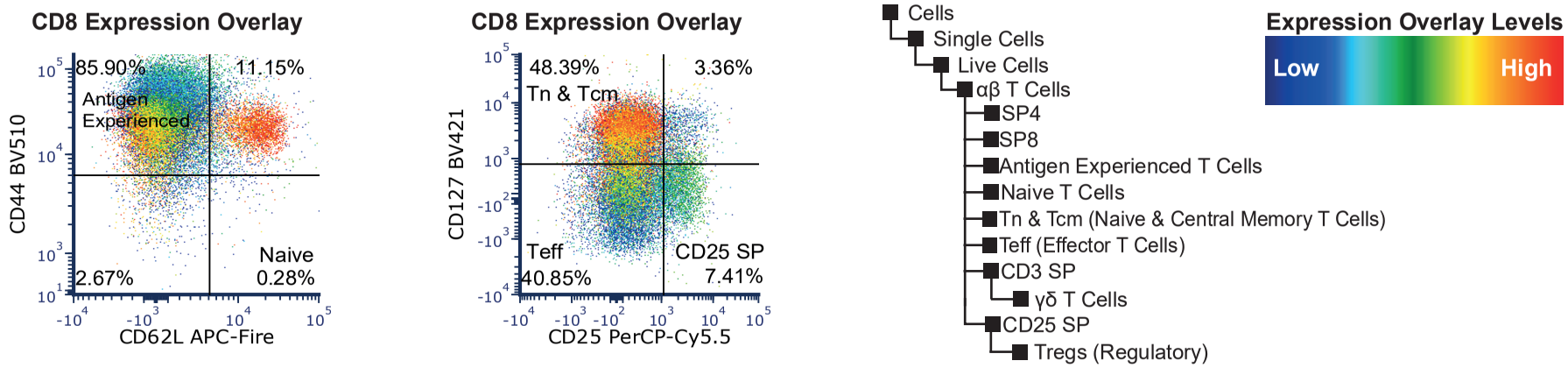


Figure 6.26. Sick iTEC-MultiRTOC recipient spleen T cell analysis: 17 weeks post-transplantation. FACS plots and gating hierarchy for iTEC recipient spleen T cells 17 weeks post transplantation (animal ID 501). Animal 501 had to be culled prior to experimental endpoint due to rapid weight loss. *Foxn1*$^{G/G}$ transplant recipients received three RTOC each consisting of 600,000 iTEC + 100,000 DN Thymocytes +100,000 WT MEFs.

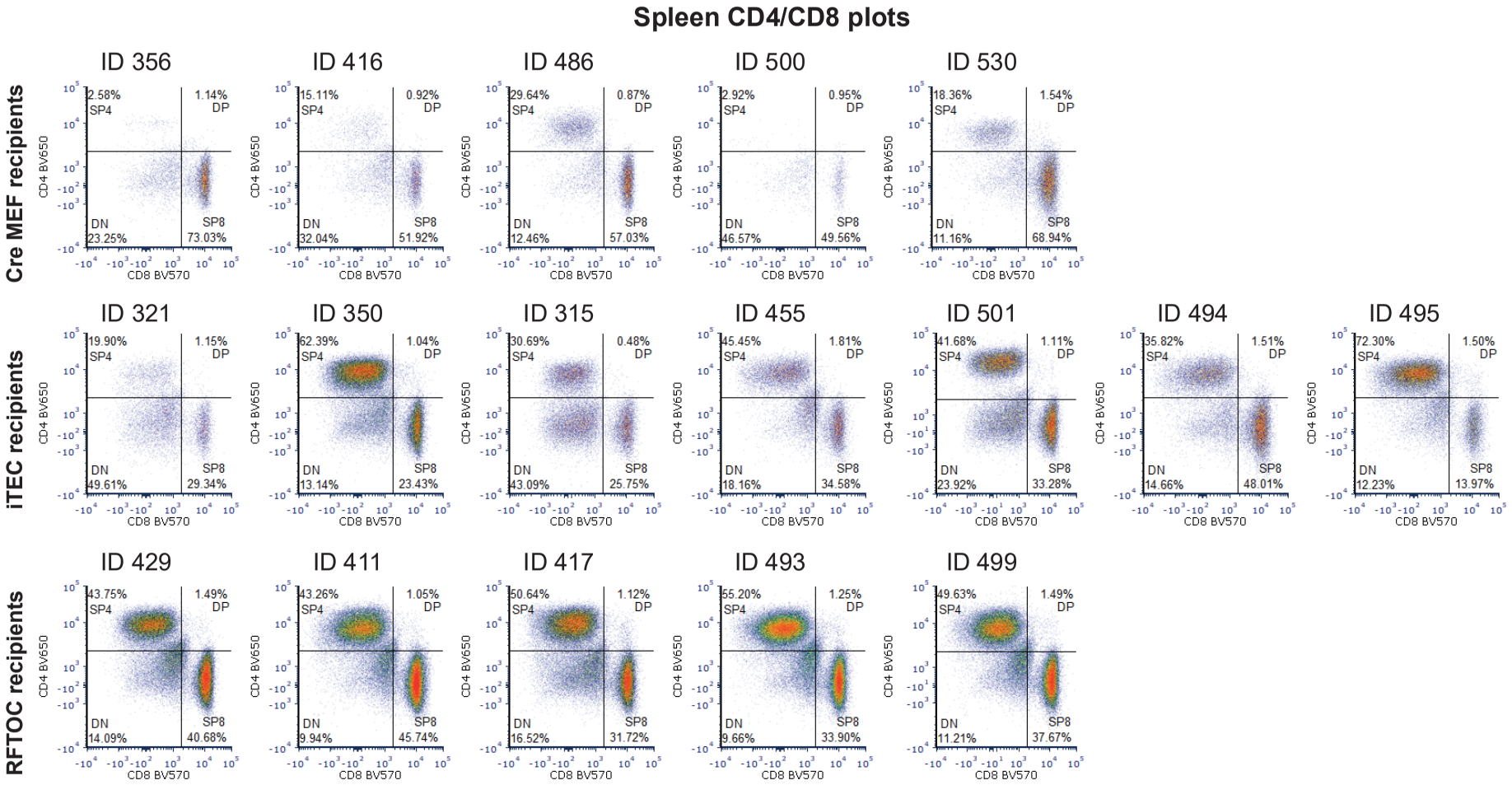
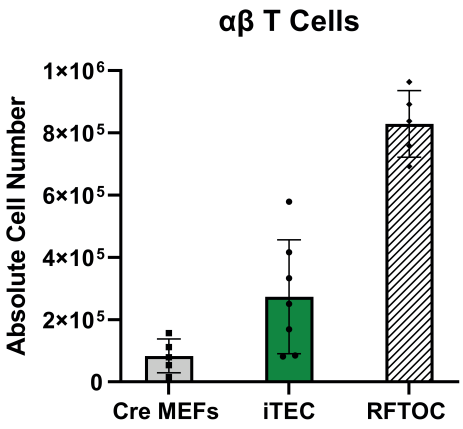


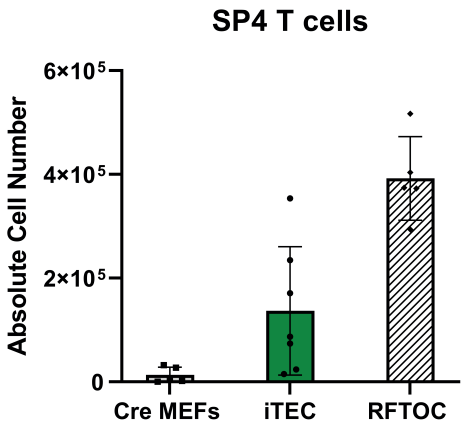
Figure 6.27. Spleen CD4/CD8 plots. Collated FACS plots of CD4 and CD8 T cell profles of *Foxn1*$^{G/G}$ recipients at experimental endpoint. Experimental endpoint is 22 weeks for all animals except ID 315 (20 weeks) and 501 (17 weeks). All plots are gated on total αβ T cells (CD3ε^{+}TCRβ^{+}).

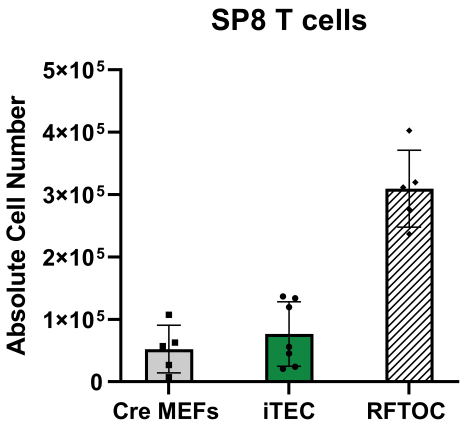
Spleen T Cell Analysis											
Condition	**Surgery Rep**	**Animal ID**	**αβ T cells**								**CD3 SP**
			Total	**SP4**	**SP8**	**Regulatory**	**Naïve**	**Naïve & central memory**	**Effector**	**Antigen Experienced**	**γδ T cells**
Cre MEFs	Rep A	**356**	79080	1550	57000	100	2390	47730	20450	27250	380
	Rep B	**416**	53510	6920	26750	170	3530	22710	15140	21930	1040
	Rep B	**486**	111680	31890	62560	270	2190	65120	29360	41590	1770
	Rep C	**500**	14880	310	7160	30	1960	5930	2920	4170	560
	Rep C	**530**	156760	27050	107240	400	6970	81870	44460	74350	990
		Mean	**83182 ± 48556**	**13544 ± 13281**	**52142 ± 34164**	**194 ± 130**	**3408 ± 1861**	**44672 ± 27548**	**22466 ± 13936**	**33858 ± 23523**	**948 ± 481**
iTEC	Rep A	**321**	84890	14750	23770	1470	2880	27640	29960	55130	2160
	Rep A	**350**	578500	353730	133630	3890	27080	278380	216000	374980	2010
	Rep A	***315**	81460	23892	20736	96	3616	18268	55212	53592	424
	Rep B	**455**	168580	73800	55680	390	3210	66140	52400	109590	3860
	Rep B	***501**	416390	170750	136750	300	3530	200570	170670	337850	5500
	Rep C	**494**	250800	87010	119360	1930	14670	142390	55630	134820	1520
	Rep C	**495**	333260	234350	45340	2690	25130	145560	103680	86750	2390
		Mean	**273411 ± 169449**	**136897 ± 114530**	**76467 ± 47847**	**1538 ± 1306**	**11445 ± 10052**	**125564 ± 88070**	**97650 ± 64996**	**164673 + 124531**	**2552 ± 1534**
RFTOC	Rep A	**429**	692680	293840	276240	1980	68490	368460	211920	327010	6530
	Rep B	**411**	891560	372990	402570	2220	148750	535790	221660	288670	8100
	Rep B	**417**	758320	374060	236560	1980	149700	365470	272320	251600	3940
	Rep C	**493**	963820	516830	319800	10890	155260	505910	292740	316780	9520
	Rep C	**499**	837890	403490	311340	9740	99070	451500	231720	349530	7170
		Mean	**828854 ± 95653**	**392242 ± 392242**	**309302 ± 55132**	**5362 ± 4061**	**124254 ± 34504**	**445426 ± 69537**	**246072 ± 31097**	**306718 ± 33792**	**7052 ± 1852**

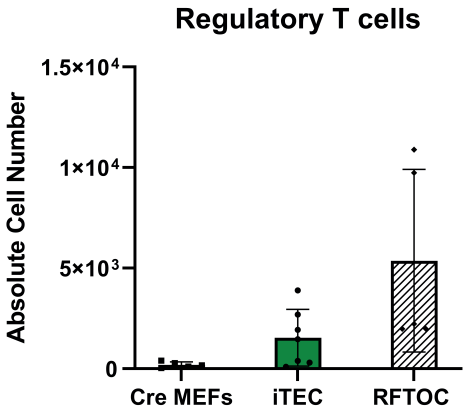
Table 6.7. Absolute numbers of spleen T cells at the experimental endpoint. Mean ±SD.

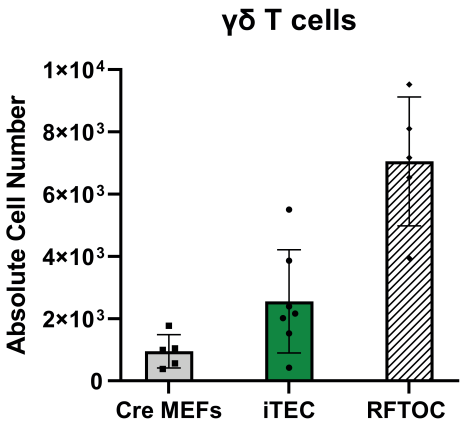
Spleen T Cell Analysis

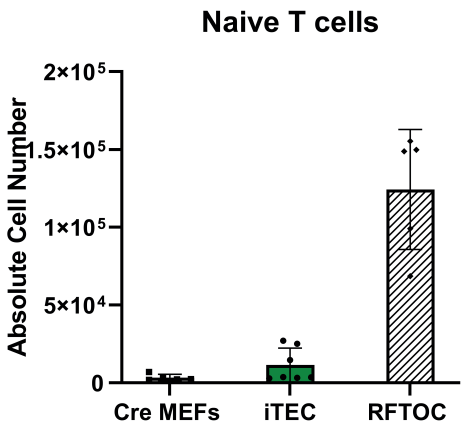












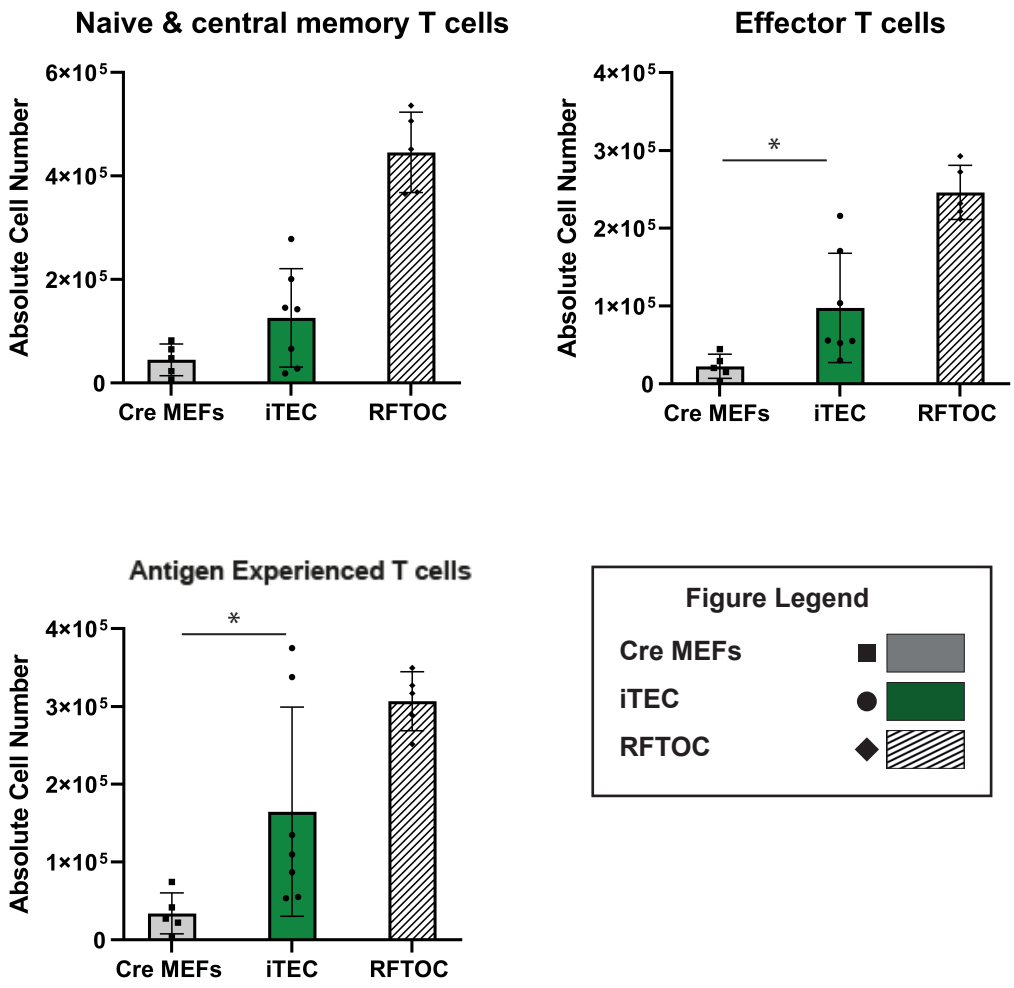


Figure 6.28. Absolute cell numbers of T cells present in spleen of *Foxn1*$^{G/G}$ transplanted mice at experimental endpoint. Each datapoint represents one animal. αβ T cells (CD3ε$^+$TCRβ$^+$), SP4(CD4$^+$), SP8 (CD8$^+$), Regulatory (CD4$^+$FOXP3$^+$), γδ T cells (CD3ε$^+$TCRγδ$^+$), Naive (CD44$^-$CD62L$^+$), Naive & central memory (CD25$^-$CD127$^+$), Effector (CD25$^-$CD127$^-$), Antigen Experienced (CD44$^+$CD62L$^-$). Spleen T cells were analysed. Absolute T cell numbers were normalised to reflect total numbers in whole spleen. Graphs display mean±SD. Cre MEFs 3 x (600,000 Cre MEFs + 100,000 WT MEFs + 100,000 DN Thymocytes), iTEC 3 x (600,000 iTEC + 100,000 WT MEFs + 100,000 DN Thymocytes), RFTOC (6 dissociated lobes + 200,000 WT MEFs). Experimental endpoint was 22 weeks post-transplantation with the exception of two animals from the iTEC condition that had to be culled at 20 and 17 weeks due to rapid weight loss and deteriorated health. N=3 independent biological experiments.

Spleen T Cell Analysis					
Condition	**Surgery Rep**	**Animal ID**	**SP4**	**SP8**	**SP4:SP8 ratio**
Cre MEFs	Rep A	**356**	1550	57000	0.03
	Rep B	**416**	6920	26750	0.26
	Rep B	**486**	31890	62560	0.51
	Rep C	**500**	310	7160	0.04
	Rep C	**530**	27050	107240	0.25
		Mean	**13544**	**52142**	**0.22**
iTEC	Rep A	**321**	14750	23770	0.62
	Rep A	**350**	353730	133630	2.65
	Rep A	***315**	23892	20736	1.15
	Rep B	**455**	73800	55680	1.33
	Rep B	***501**	170750	136750	1.25
	Rep C	**494**	87010	119360	0.73
	Rep C	**495**	234350	45340	5.17
		Mean	**136897**	**76467**	**1.84**
RFTOC	Rep A	**429**	293840	276240	1.06
	Rep B	**411**	372990	402570	0.93
	Rep B	**417**	374060	236560	1.58
	Rep C	**493**	516830	319800	1.62
	Rep C	**499**	403490	311340	1.30
		Mean	**392242**	**309302**	**1.30**

Table 6.8. CD4:CD8 ration of spleen T cells at experimental endpoint.

6.3. Analysis of the T cell repertoire in iTEC-RTOC recipients

6.3.A. Interpretation of library quality

In order to determine whether grafted iTEC-RTOC produce a T cell repertoire of normal diversity, I performed a TCRβ repertoire RNA-sequencing analysis of mice that had received iTEC-MultiRTOC (without fetal thymic mesenchyme), or a control RFTOC graft twenty-two weeks previously (with the exception of the sick iTEC recipients which were culled prematurely as stated previously). In brief, TCRβ V-region (Vβ) amplicons were generated for each recipient, to examine the clonal structure of the spleen and lymph node T populations and hence to determine TCR diversity (see Figure 6.29). TCRβ amplicons were generated from spleen and lymph node T cells, separately, for all animals from the three independent experiments in which grafts were transplanted into *Foxn1*$^{G/G}$ hosts. The cDNA synthesis failed for RFTOC samples 429(Spleen), 493(Spleen), Cre MEF samples 356(Lymph Nodes) and iTEC-RTOC samples 501(Lymph Nodes), 350(Lymph Nodes), 455 (Lymph Nodes) (number indicates animal ID).

All samples were pooled and sequenced using Illumina MiSeq v2 Next Generation Sequencing technology. Bioinformatics analysis was carried out by Natasha Smith and Dr. Graeme Cowan (School of Biological Sciences, University of Edinburgh). Each amplicon contained a unique barcode for each sample (Index primer) and Unique Molecular Identifiers (UMI) which uniquely tag each transcript to allow multiplexing of samples for MiSeq sequencing and to increase the precision of calculating the number of clones in a repertoire, respectively.

Table 6.9 shows the UMI and unique CDR3 read counts obtained for each sample following sequencing. It would be expected that a diverse TCR repertoire would contain a high number of both UMI and unique CDR3 read counts (as observed for the positive control RFTOC recipients). In contrast, a high UMI count combined with a low unique CDR3 count would indicate a low diversity. However, in these experiments, the starting number of T cells was not consistent for each library. Instead, samples were prepared from 10^6 whole CD45$^+$ enriched spleen or lymph node cells. Therefore, the samples were not generated from the same number of T cells each time, and a low UMI count may indicate a low T cell input at the start of the sequencing protocol. Ideally, the samples would all have been prepared from the same number of CD3ε$^+$TCRβ$^+$ T cells, eliminating amplification bias based on input material.

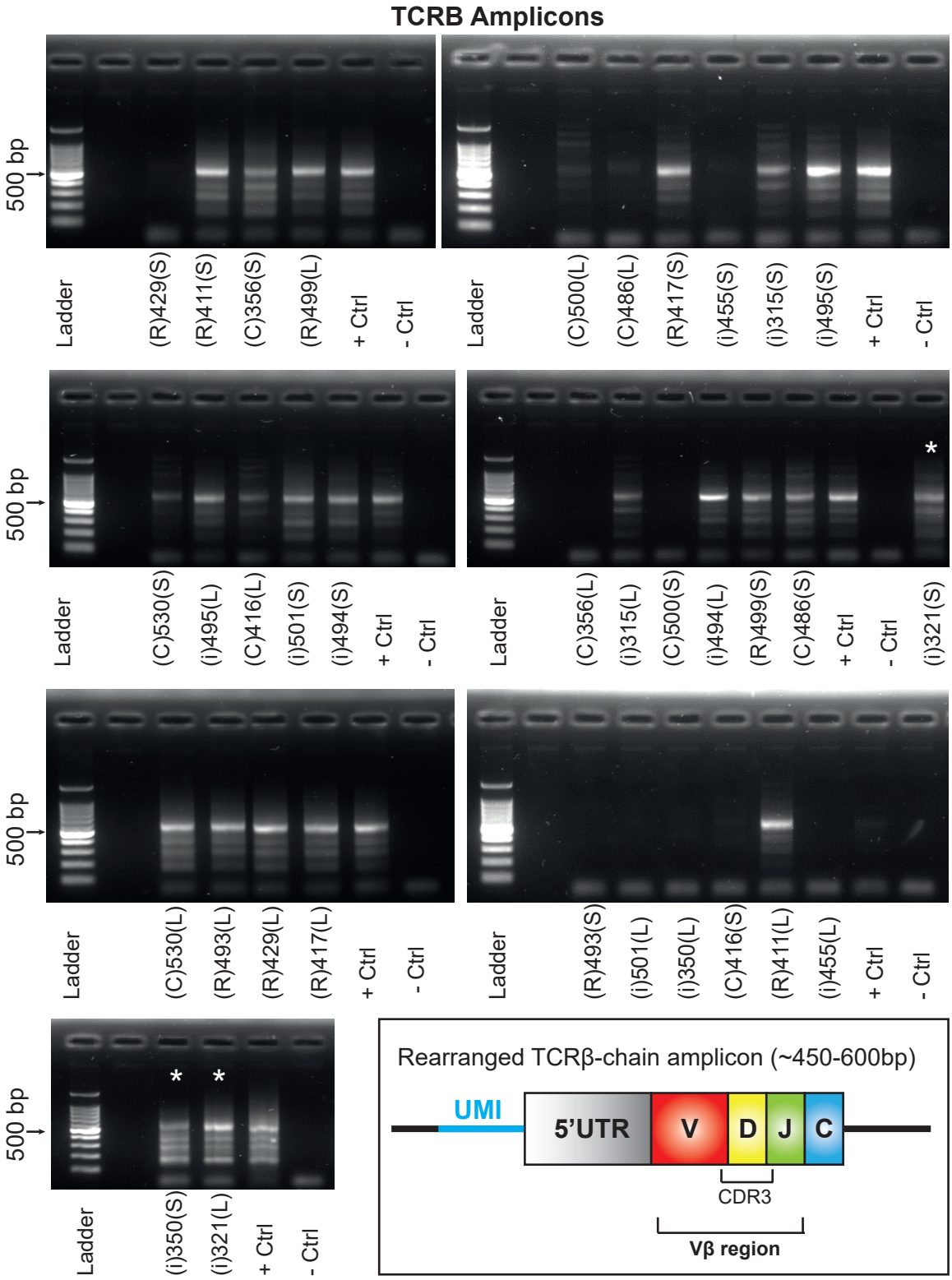


Figure 6.29. TCRB amplicon PCR gels. Sample name is in the format: [(Condition: C for Cre, i for iTEC and R for RFTOC)AnimalID(S for Spleen/L for Lymph Nodes)]. All samples underwent 25 cycles of amplification with the exception of samples indicated with a white asterisk which underwent 30 cycles due to initial dim product. Bottom right: illustration of TCRB amplicons generated for each sample. Each sample has a unique index barcode (not depicted) and each transcript has a unique molecular identifier (UMI) barcode for de-multiplexing samples during bioinformatic analysis following RNA-Seq. (R)429(S), (C)356(L), (R)493(S), (i)501(L), (i)350(L), (i)455(L) failed TCR amplicon generation and amplification. (i)455(S), (C)500(S), (C)416(S) very dim amplicon.

Sample Name	Unique UMIs	Unique CDR3s	UMI:CDR3	No. αβ T cells
Cre_356_LN*	no reads	no reads	-	98826
Cre_356_Spleen	2516	475	5.3	79080
Cre_416_LN	444	209	2.1	95410
Cre_416_Spleen	25	22	1.1	53510
Cre_486_LN	77	47	1.6	125622
Cre_486_Spleen	2370	455	5.2	111680
Cre_500_LN	75	56	1.3	30352
Cre_500_Spleen *	3	3	1	14880
Cre_530_LN	4851	462	10.5	245028
Cre_530_Spleen	309	67	4.6	156760
Mean	**1333**	**224**	**6**	**110696**
iTEC_315_LN	579	160	3.6	246084
iTEC_315_Spleen	399	104	3.8	81460
iTEC_321_LN	126	76	1.6	681960
iTEC_321_Spleen	62	42	1.5	84890
iTEC_350_LN	18	13	1.4	480326
iTEC_350_Spleen	78	54	1.4	578500
iTEC_455_LN *	0	0	0	291046
iTEC_455_Spleen	33	28	1.2	168580
iTEC_494_LN	1996	535	3.7	257430
iTEC_494_Spleen	2834	563	5	250800
iTEC_495_LN	2806	1222	2.3	302138
iTEC_495_Spleen	1966	961	2	333260
iTEC_501_LN *	2	2	1	86130
iTEC_501_Spleen	4325	494	8.7	416390
Mean	**1269**	**354**	**3.6**	**323485**
RFTOC_411_LN	1469	1336	1.1	670520
RFTOC_411_Spleen	8845	5962	1.5	891560
RFTOC_417_LN	7533	6161	1.2	912860
RFTOC_417_Spleen	1233	987	1.2	758320
RFTOC_429_LN	10187	6398	1.6	823269
RFTOC_429_Spleen	44	42	1.04	692680
RFTOC_493_LN	4648	3540	1.3	779430
RFTOC_493_Spleen*	no reads	no reads	-	963820
RFTOC_499_LN	4168	2969	1.4	487375
RFTOC_499_Spleen	3366	2314	1.4	837890
Mean	**4610**	**3301**	**1.4**	**761545**

Table 6.9. UMI and CDR3 counts RNA-Seq counts. Samples with * were not included in mean calculations. Samples highlighted in blue represent sick animals culled early. Number of αβ T cells represents total number of αβ T cells found in the whole spleen or in seven lymph nodes of the animal.

Figure 6.30 shows plots of UMI versus unique CDR3 count for each sample in all three conditions: RFTOC, Cre MEF and iTEC recipients. A line of best fit based on linear regression analysis was incorporated based solely on the positive control RFTOC samples, as it is expected that these animals have a diverse repertoire. The samples closest to the line of best fit exhibit the most repertoire diversity.

From the iTEC samples 494_Spleen and 494_LN and 495_Spleen and 495_LN appear closest to the line of best fit, with the rest of the samples presenting with low UMI and CDR3 counts.

Mouse 501_Spleen, despite having a high UMI count, had a low unique CDR3 count, indicating the possibility of clonal expansion of specific T cells. This is of interest as this animal had to be culled prematurely due to rapid weight loss possibly due to autoimmunity, although presence of autoantibodies and histological analysis for immune infiltrates was not examined at this stage. In the case of autoimmunity, clonal expansion of autoimmune clones would be expected.

Interestingly, among the negative controls, Cre samples 485_Spleen, 356_Spleen and 530_LN had higher UMI and CDR3 counts than the rest of the negative control samples. However, compared to iTEC recipients 494 and 495, they have decreased unique CDR3 counts and are at a greater distance from the line of best fit, indicating that the increased UMI counts represent clonally expanded T cells.

In order to determine whether the low UMI counts observed in some samples could be due to low T cell numbers, and therefore indicate poor amplification rather than low diversity of transcripts, UMI counts were plotted against the corresponding T cell numbers found in mice (Figure 6.31, top panel). If UMI and T cell count were proportional to each other, this would indicate that a low UMI count most likely reflects a technical issue in library preparation rather than low TCR diversity. It is important to note that Cre recipients are expected to have lower UMI counts as they have very low numbers of T cells (that are extrathymically derived) compared to iTEC and RFTOC recipients.

From the data however, it does not appear that UMI is correlated to T cell counts of the mice. All groups display a wide range of UMI counts even at low T cell numbers. The same analysis was performed for unique CDR3 counts against absolute T cell counts present in recipients (Figure 6.31, bottom panel). Similarly, increased T cell counts did not necessarily result in increased unique CDR3 counts. This may

indicate clonal expansion of specific clonotypes in samples with high T cell numbers but low CDR3 counts.

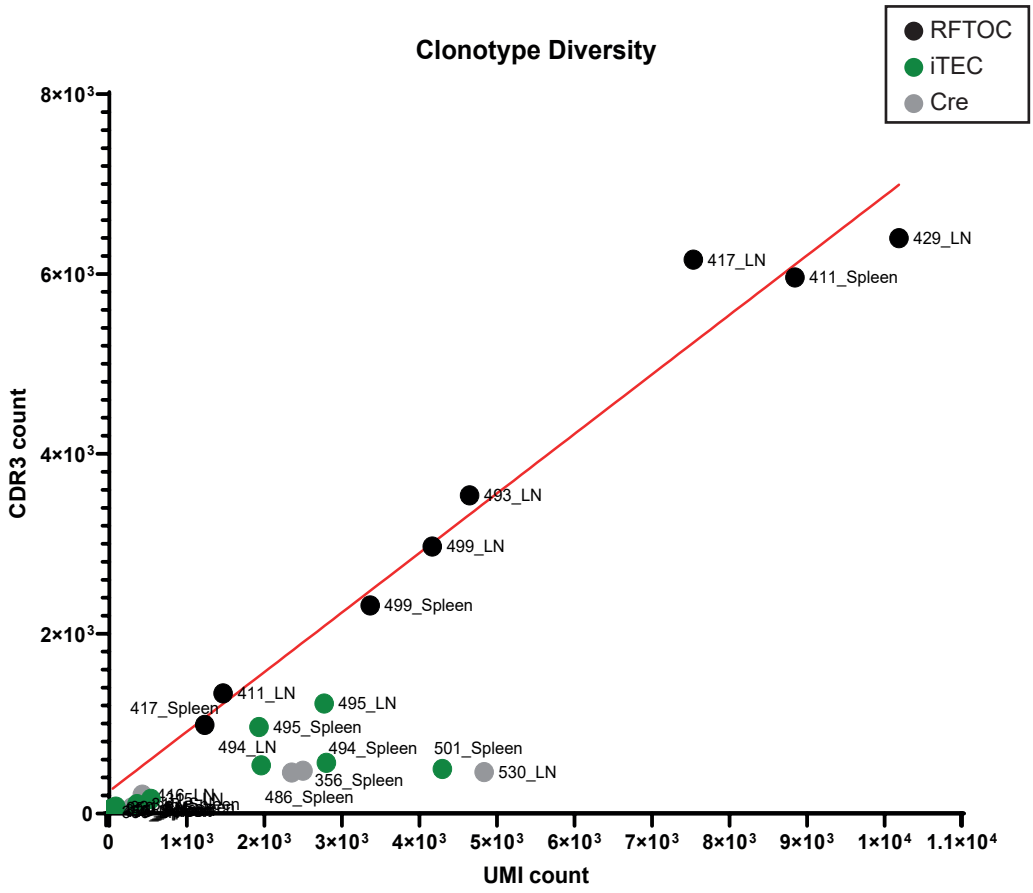


Figure 6.30. UMI vs CDR3 count present in each sample. Unique Molecular Identifier (UMI) counts represent number of unique transcripts present in each sample library prior to amplification and CDR3 count represents number of unique CDR3 variable sequences present per sample. The red line represents line of best fit with simple linear regression for the positive control RFTOC dataset in order to show trend of UMI:CDR3 count in a diverse TCR pool.

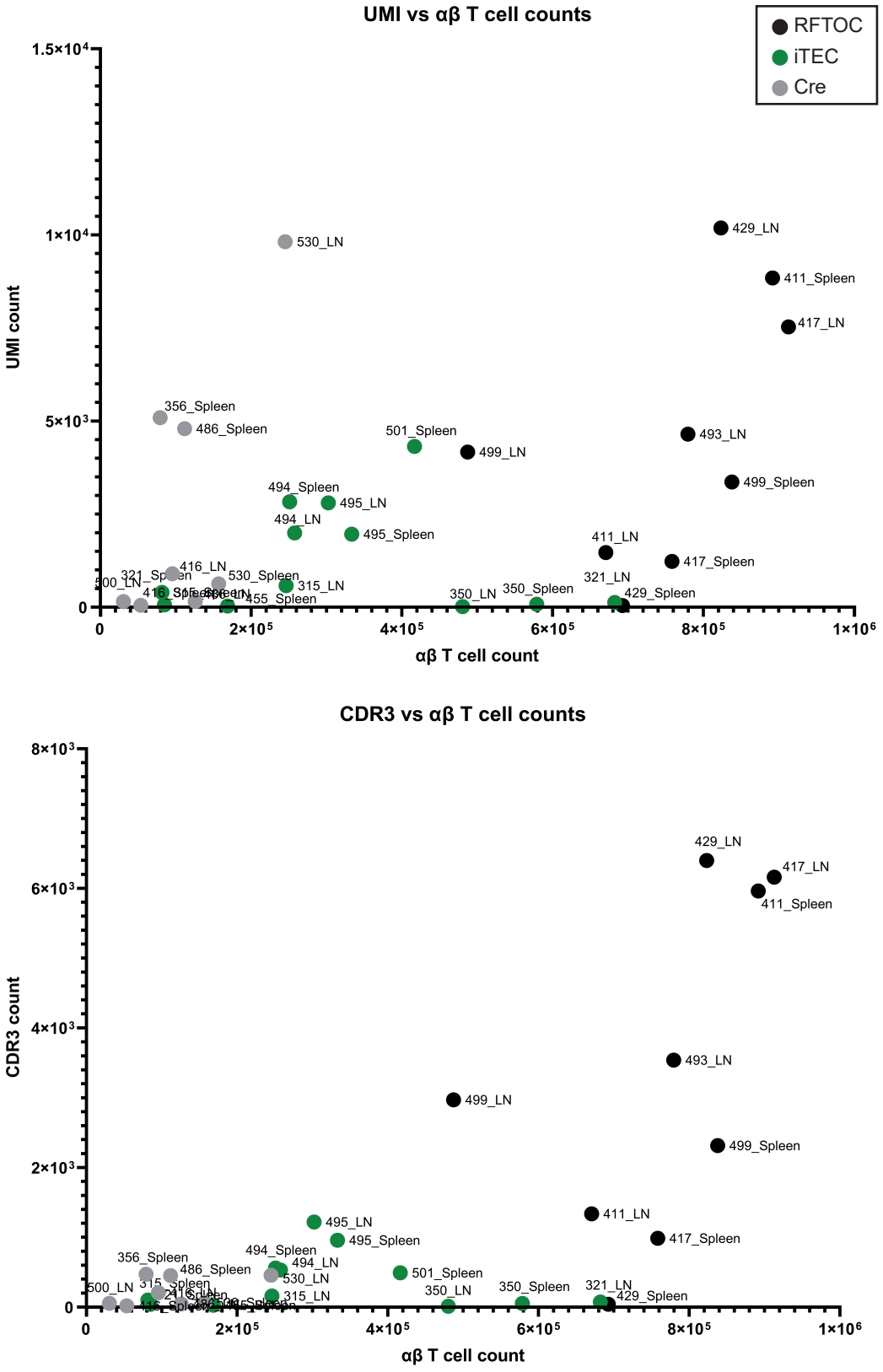


Figure 6.31. Correlating libraries to T cell count in samples. Unique Molecular Identifiers (UMIs) counts represent number of unique transcripts prior to amplification present in each sample library and CDR3 count represents number of unique CDR3 variable sequences present per sample. These were compared against total αβ T cell ($CD3\varepsilon^{+}TCR\beta^{+}$) counts present whole spleen or seven lymph nodes of the recipient.

6.3.B. Heatmap clustering for TCR diversity

The V region sequencing data described above were normalised using the MiGEC (molecular identifier groups-based error correction) tool, which allows error correction while simultaneously preserving diversity of immune repertoires when UMIs are utilised (Shugay *et al*., 2014). This is essential for removal of PCR and sequencing errors associated with deep repertoire analysis (Bolotin *et al*., 2012). Following this data normalisation, a heatmap was generated depicting the frequency (%) of the common T Cell Receptor Beta Variable (TRBV) gene sequence clonotypes present within each sample (Figure 6.32). Samples which did not generate any reads (356_LN, 429_Spleen, 455_LN), had very low UMI count (416_Spleen, 501_LN), or did not meet MiGEC criteria (350_LN, 411_LN, 493_Spleen) were excluded from the next step of the analysis.

In this analysis, the Cre-MEF recipient samples broadly clustered together, and samples from three of the iTEC-RTOC recipients (315, 350, 501) also clustered closely with the Cre-MEFs samples (Figure 6.32). Note that mice 315 and 501 had to be culled early due to rapid weight loss. Similarly, the RTOC recipient samples all clustered together. Of the remaining iTEC-RTOC recipient samples, two (494, 495) clustered closely with the RFTOC samples, and one (321) clustered between the RFTOC and Cre-MEF samples (Figure 6.32).

All of the positive control RFTOC samples had a wide range of TRBV clones, with no clonotypes being overrepresented, indicating TCR diversity. Recipients 494 and 495, which received iTEC-RTOC grafts in the same experiment and were generated from the same batch of iTEC, clustered with the positive control RFTOC samples, indicating that these recipients had established a diverse TCR repertoire. The lymph node and spleen samples of each of these iTEC-RTOC recipients (494, 495) also clustered together, indicating the robustness of this result. Although these samples clustered with the RFTOC condition and exhibited a wide distribution of Vβ regions, they did contain some clones that were represented at a higher frequency than others. iTEC-495LN and iTEC-494LN both had a high frequency of the TRBV5 clonotype (>15%) whereas iTEC-495Spleen had a high percentage of TRBV13-2 (~15%) and iTEC-494Spleen of TRBV13-3 (>15%).

The diversity of clonotypes within the Cre-MEF/iTEC-RTOC cluster varied, however, most samples contained a small set of clonotypes that were highly overrepresented. Although the negative control Cre MEF grafts did not support normal thymopoiesis

(as shown in above) some extrathymically derived T cells accumulated over time, as seen in nude mice (Troy and Shen, 2003). Another possibility is the spontaneous maturation of DNs present within the transplant. However, extrathymically derived T cells and their diversity remain poorly characterised. It would be expected that *in vivo* expansion of a few extrathymically derived clones would take place driven by homeostatic proliferation, due to the severe lymphopenic environment they are entering (Mackall *et al*., 1996; Kieper and Jameson, 1999; Kieper *et al*., 2005).

From the negative control Cre MEF samples, Cre-500Spleen contained only 4 major clonotypes, which each occupied 25% of the repertoire. The iTEC recipient mice which were culled early due to rapid weight loss, clustered with the Cre recipients and each also exhibited a dominant clone that represented 25% of the repertoire (iTEC-315: TRBV12-1 and iTEC-501: TRBV13-2). This might indicate the presence of an activated autoreactive clone. iTEC recipients 455 and 350 also clustered with the negative controls, with recipient 321 being intermediate between the RFTOC and Cre-MEFs clusters. This suggests that not all iTEC recipients established a diverse T cell repertoire. However, the iTEC-455, -350 and -321 samples had the lowest UMI counts of all the iTEC recipients and therefore issues in library preparation cannot be excluded for accounting in not capturing a diverse repertoire if present (Table 6.9).

Overall, despite the variation in outcome, these preliminary data establish that iTEC-RTOC grafts can establish a diverse T cell repertoire similar to that supported by transplanted native thymic tissue.

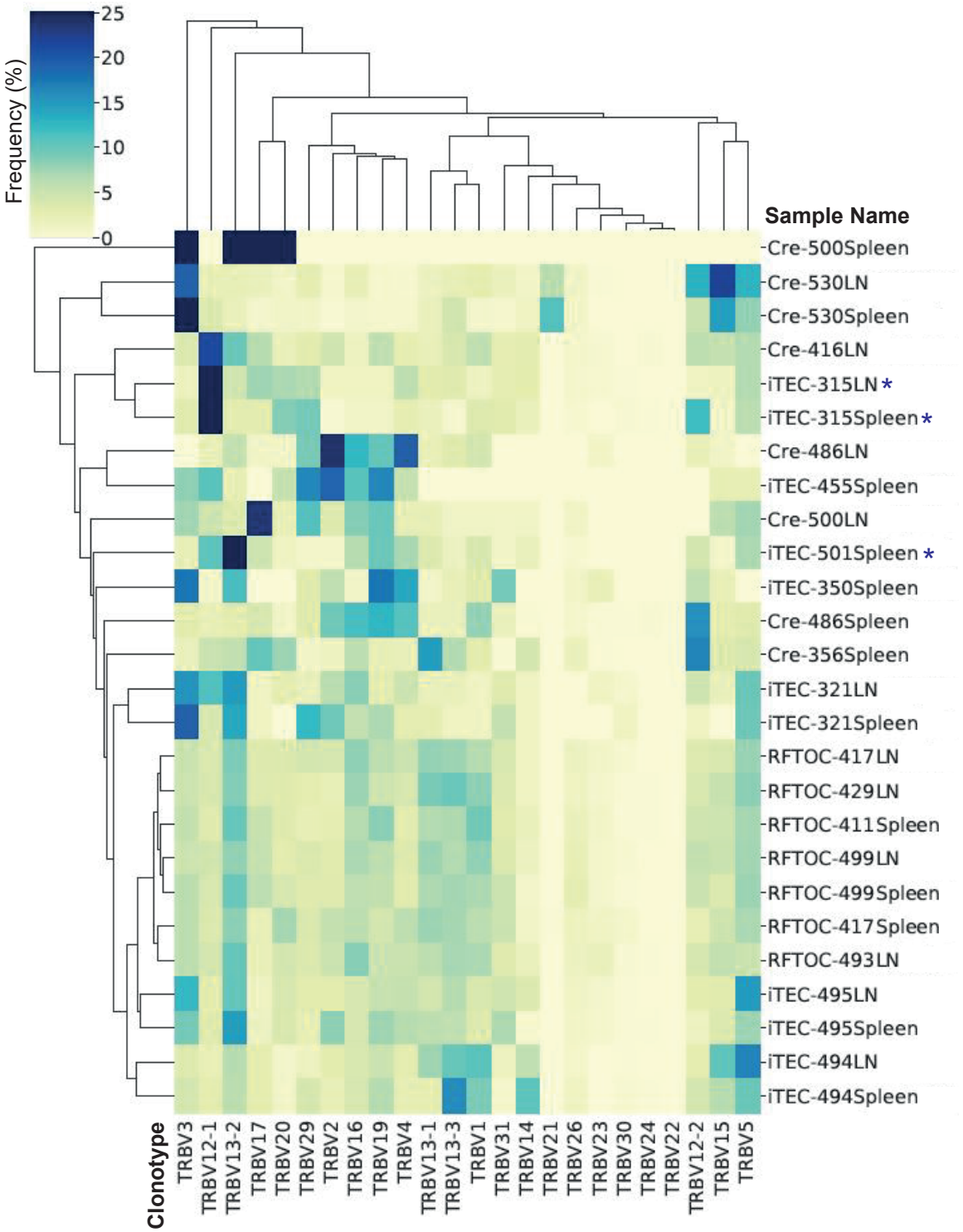


Figure 6.32. Heatmap representation of TRB variable (TRBV) genes present in transplanted *Foxn1*$^{G/G}$ mice. The heatmap depicts sample clustering per commonly found TRBV genes. Column annotations represent TRBV clonotype, while row annotation describe sample type as follows: [Condition received(iTEC/Cre/RFTOC)]-[Animal ID][Obtained from Spleen or Lymph Nodes(LN)]. Units are based on the frequency (%) of clonotypes present in each sample. Mice which were terminated early due to rapid weight loss are indicated with an asterisk *.

6.4. Investigation of the longevity of the iTEC-RTOC grafts *in vivo*

Having established that iTEC-RTOC could repopulate the periphery of *Foxn1*$^{G/G}$ hosts with a diverse TCR repertoire, I next set out to test the longevity of the the iTEC-RTOC following grafting under the kidney capsule.

Thus, the kidneys were recovered from all of the transplanted mice detailed above, and grafts were removed from under the kidney capsule from any mice where a macroscopic graft was detected. H&E analysis of graft sections are shown in Figure 6.33. The RFTOC grafts displayed characteristic cortical and medullary organization and were tightly packed with thymocytes. None of the recovered iTEC-RTOC grafts, or Cre MEFs graft sites, exhibited this structure or cellularity, although the iTEC-RTOC grafts recovered at earlier timepoints (two and four weeks) had exhibited characteristic thymus morphology (as discussed in Chapters 3, 4).

In order to characterize the cells present in the grafts, immunohistochemical (IHC) staining was carried out using the following markers: cytokeratin 8 (K8: green), an epithelial cell marker associated with cTEC (Klug *et al*., 1998, 2002; Danzl *et al*., 2014), cytokeratin 14 (K14: magenta) and Ulex Europaeus Agglutinin-1 (UEA1: gray) both associated with mTEC (Klug *et al.*, 1998; Kuraguchi *et al*., 2006; Lomada *et al*., 2007; Ulyanchenko *et al*., 2016) (Figure 6.34). IHC staining is shown for two different mice for each condition. All grafts were recovered at twenty-two weeks except for animal ID 501 which was recovered at seventeen weeks post-transplantation.

As expected, the RFTOC grafts displayed staining characteristic of the native thymus: reticular staining with K8, consistent with the presence of cortical regions, and medullary regions co-expressing K14 and UEA1. The Cre MEF grafts did not stain with K8, K14 or UEA1; note that the fluorescence evident in the green channel is background.

The iTEC grafts exhibited small regions of K8^{+} epithelial cells but did not stain with K14 or UEA1. Since characteristic cortical and medullary region staining was evident at earlier timepoints (Chapter 3 and Chapter 4), this indicated that only some iTEC cells were able to survive for twenty-two weeks *in vivo*. In animal ID 321, the gray staining represents kidney cells adjacent to the graft region, evident from their morphology. Renal tissue displays significant autofluorescence and was removed from the grafts before sectioning as far as possible (Zhang *et al*., 2018).

The cTEC functional marker β5t (green) was used to further test the cTEC identity of the TEC-like cells that remained in the iTEC grafts (Figure 6.35). The recovered RFTOC grafts displayed strong β5t staining in regions of dense cellularity (as seen by DAPI staining) characteristic of the thymic cortex, with no β5t staining observed in the medullary regions (less dense $DAPI^+$ regions). The Cre MEF grafts did not display any strong β5t staining. Among the iTEC-RTOC graft recipients, animals 501, 321 and 350 all contained $β5t^+$ cells, indicating the cTEC identity of the $K8^+$ iTEC. The graft recovered from animal 494 did not display β5t staining. The iTEC grafts also contained $K8^-$ or $β5t^-$ cells, suggesting the presence of other cell types, most likely derived from the mesenchymal (WT MEFs) or haematopoietic (thymocytes) that were included in RTOC prior to transplantation or recruited from the host (eg. vascular endothelium).

The grafts were also stained with Vimentin (Vim: green, a type III intermediate filament that stains mesenchymal cells (Wu *et al*., 2018)), and CD45 (magenta), to determine whether cells present in grafts, that did not stain with K8, K14, UEA1 or β5t, were mesenchymal or haematopoietic cells (Figure 6.36). The recovered RFTOC grafts were densely packed with $CD45^+$ cells characteristic of thymocyte staining in the native thymus. Additionally, Vim^+ cells were found throughout the positive control grafts and in medullary regions where fibroblasts are known to play a role in central tolerance (Nitta *et al*., 2020). This colocalisation can be seen in the graft recovered from mouse 417.

The negative control Cre MEF grafts stained with vimentin, as expected, and displayed very few cells of haematopoietic origin. As expected from H&E analysis, which revealed similarities between Cre MEF and iTEC grafts recovered twenty-two weeks post-grafting, the majority of the cells present in iTEC grafts were mesenchymal (presumably derived from WT MEFs or kidney mesenchyme) rather than surviving iTEC. Few $CD45^+$ were present, signifying that thymopoiesis was no longer taking place within the grafts.

To conclude, iTEC grafts recovered after seventeen weeks or more *in vivo* contained very few cTEC and no mTEC, indicating that the iTEC grafts generated under these conditions have a limited lifespan. This suggests that iTEC may not generate the TEC progenitor(s) required to replenish TEC as they turn over, or that they may not establish the niche required to maintain such a TEC progenitor compartment.

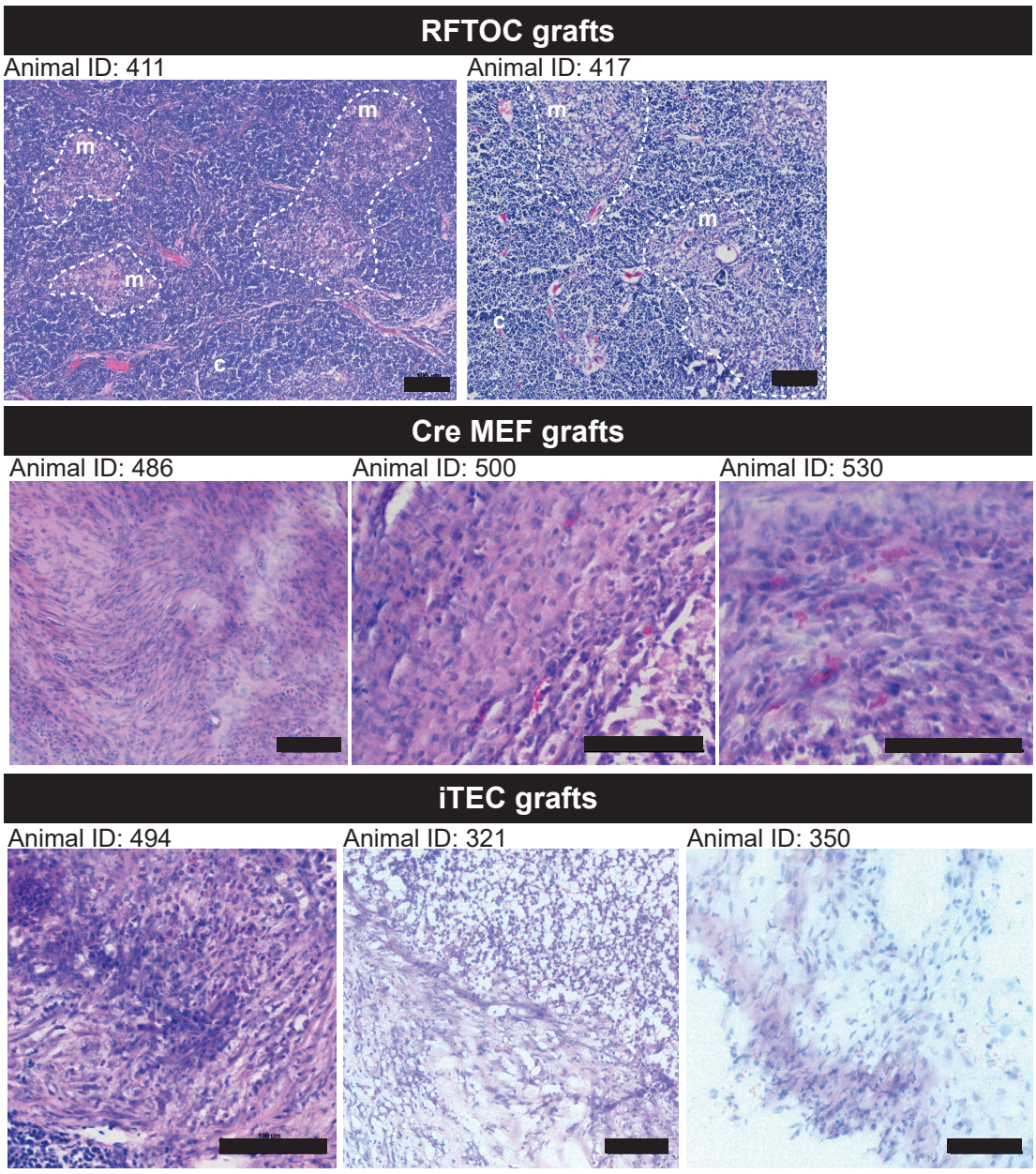


Figure 6.33. Histology of recovered grafts 22 weeks *in vivo*. Haematoxylin and eosin (H&E) staining of sectioned recovered grafts for RFTOC, Cre MEF and iTEC conditions recovered 22 weeks post-transplantation. Cortical areas (c) are classified with dense hematoxylin staining (purple) as an indication of an abundance of thymocytes. Medullary areas (m) are classified with dense eosin staining (pink) as an indication of less thymocytes present. Medullary areas in RFTOC grafts are outlined with a white dotted line. Each image represents a separate animal. Cre MEF and iTEC grafts display absence of medullary regions. Scale bar 100 μm.

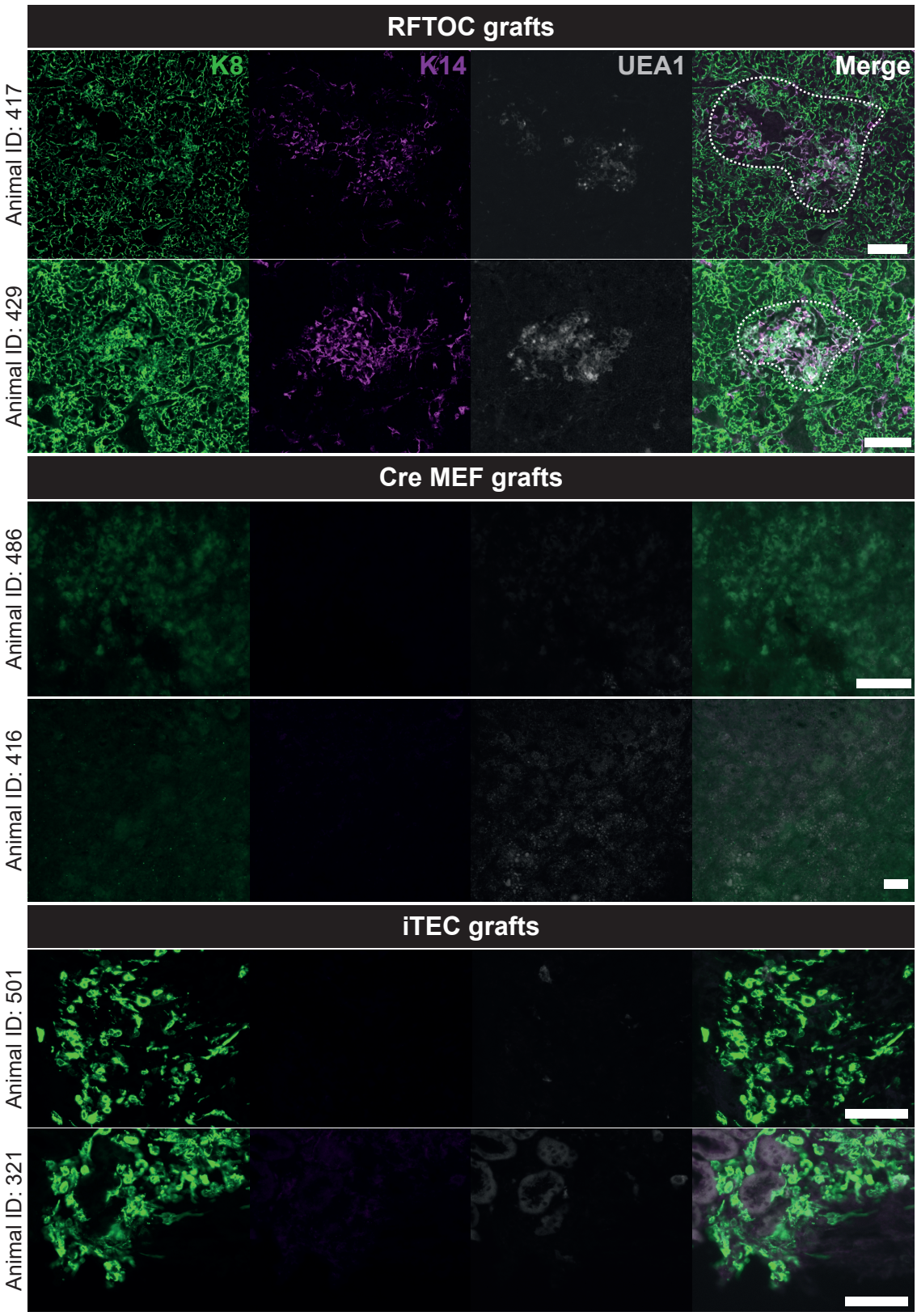


Figure 6.34. Immunohistochemistry staining of recovered grafts from *Foxn1*$^{G/G}$ transplant recipients. Grafts were recovered at 22 weeks with the exception of animal ID 501 which was recovered at 17 weeks. Graft cryosections were stained with cytokeratin 8-K8 (green) for cortical regions, cytokeratin 14-K14 (magenta) and UEA1 (grayscale) for medullary regions. Medullary areas in RFTOC grafts are outlined with a white dotted line. Each image represents a separate animal. RFTOC (6 dissociated whole E15.5 lobes + 200,000 WT MEFs), iTEC 3 x (600,000 iTEC + 100,000 DN thymocytes + 100,000 WT MEFs), Cre MEFs 3 x (600,000 Cre MEFs + 100,000 DN thymocytes + 100,000 WT MEFs). Scale bar 100 μm.

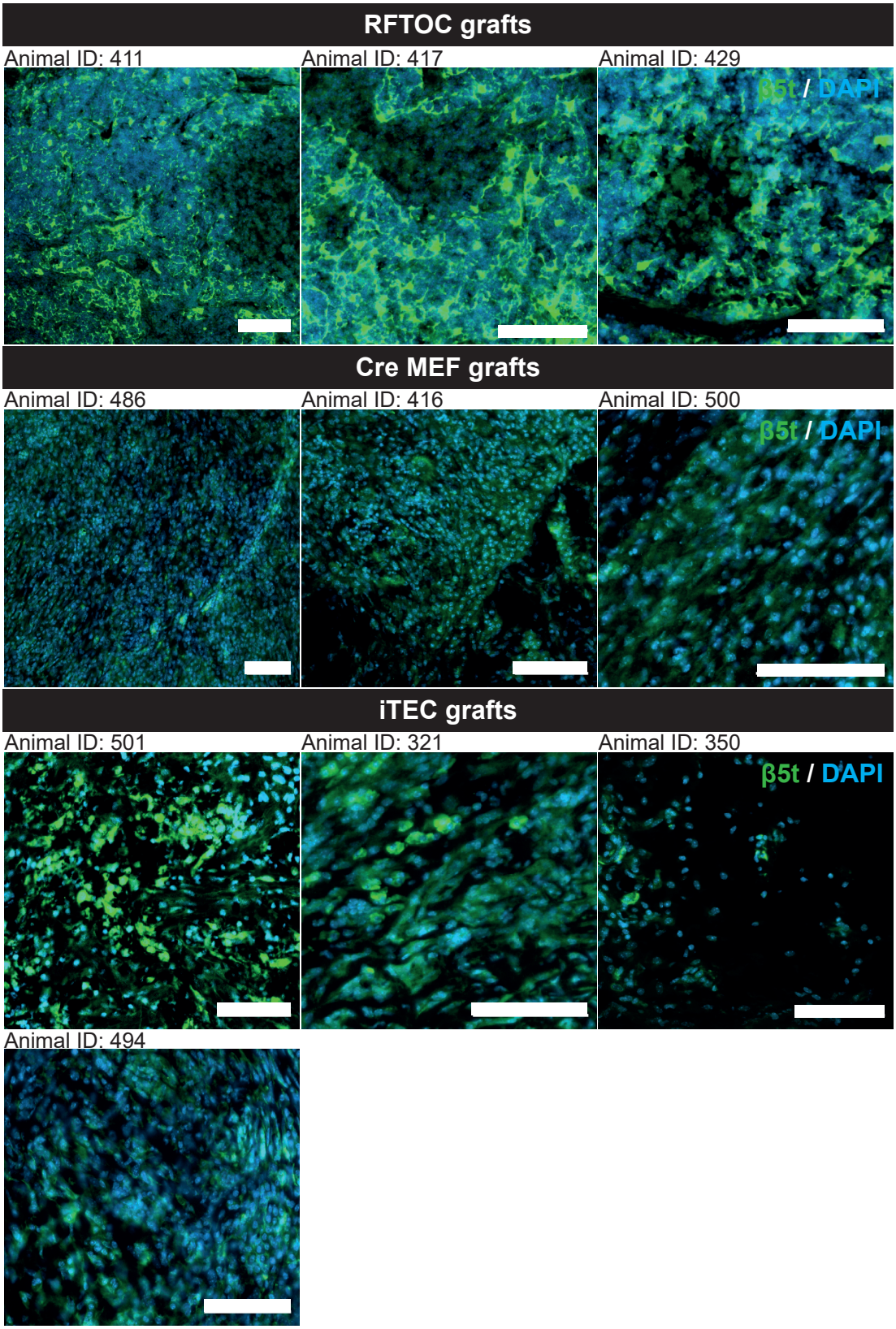
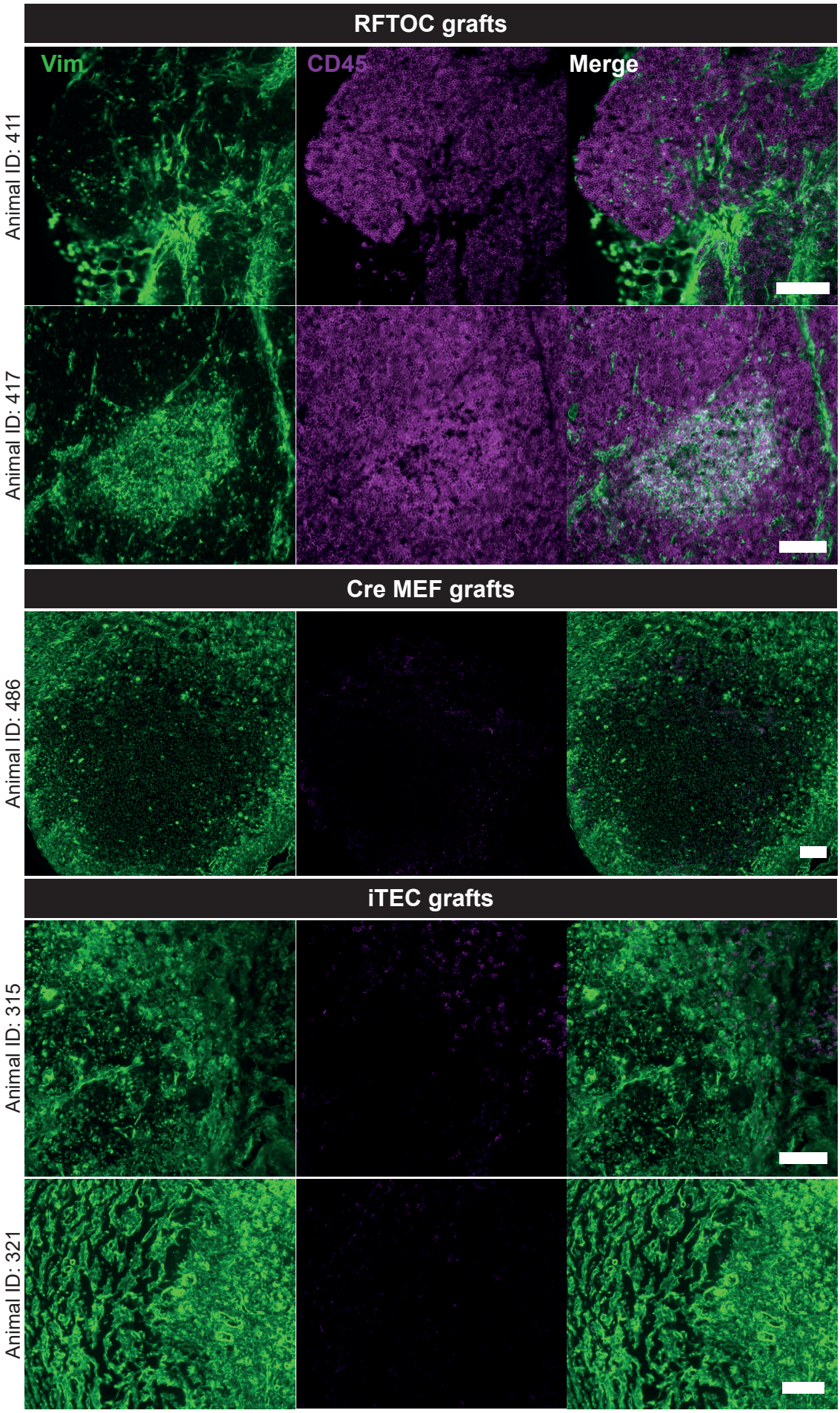


Figure 6.35. β5t staining of recovered grafts from *Foxn1*$^{G/G}$ transplant recipients. Grafts were recovered at 22 weeks with the exception of animal ID 501 which was recovered at 17 weeks. Graft cryosections were stained with β5t cortical TEC marker (green) and nuclear cell marker DAPI (blue). Each image represents a separate animal. RFTOC (6 dissociated whole E15.5 lobes + 200,000 WT MEFs), iTEC 3 x (600,000 iTEC + 100,000 DN thymocytes + 100,000 WT MEFs), Cre MEFs 3 x (600,000 Cre MEFs + 100,000 DN thymocytes + 100,000 WT MEFs). Scale bar 100 μm.


RFTOC grafts
Vim
CD45
Merge
Animal ID: 411
Animal ID: 417
Cre MEF grafts
Animal ID: 486
iTEC grafts
Animal ID: 315
Animal ID: 321

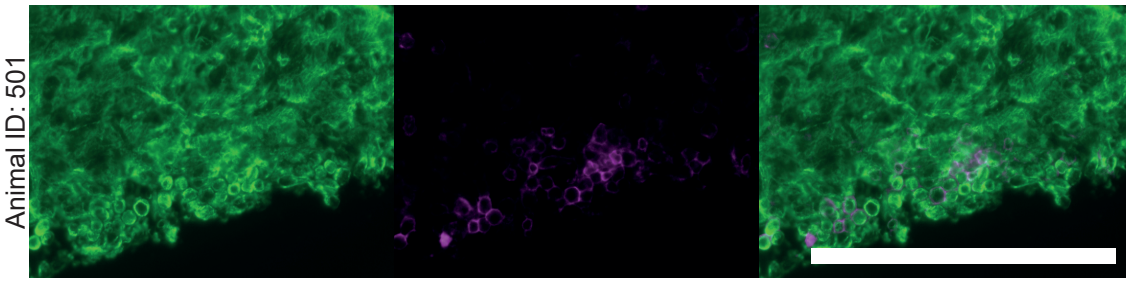


Figure 6.36. Vimentin and CD45 staining of recovered grafts from *Foxn1*$^{G/G}$ transplant recipients. Grafts were recovered at 22 weeks with the exception of animal ID 501 which was recovered at 17 weeks and animal 315 recovered at 20 weeks. Graft cryosections were stained with mesenchymal marker Vimentin (Vim: green) and haematopoietic cell marker CD45 (magenta) Each image represents a separate animal. RFTOC (6 dissociated whole E15.5 lobes + 200,000 WT MEFs), iTEC 3 x (600,000 iTEC + 100,000 DN thymocytes + 100,000 WT MEFs), Cre MEFs 3 x (600,000 Cre MEFs + 100,000 DN thymocytes + 100,000 WT MEFs). Scale bar 100 μm.

6.5. Discussion

The ability of iTEC-RTOC grafts to reconstitute the T cell compartment in *Foxn1*$^{G/G}$ recipients and establish a diverse TCR repertoire, and to survive for up to twenty-two weeks *in vivo*, was investigated.

6.5.A. iTEC-RTOC mediate T cell reconstitution of *Foxn1*$^{G/G}$ mice

6.5.A.I. Background on nude athymic mouse T cells

Athymic *nude* mice (*nu/nu*), despite lacking a thymic microenvironment, generate very low numbers of αβ and γδ T cells that increase with age. This T cell development is thought to occur extrathymically. Although the mechanisms with which such T cells arise are poorly understood, this is thought to primarily occur in the liver and intestine (Abo, 2001). Even in immunocompetent mice, presence of extrathymic T cells have been reported and expand with age concurrently to thymic involution (Abo, 2001).

Several studies have noted the accumulation of extrathymically derived αβ T cells, which display antigen-specific activity, in the spleen and lymph nodes of ageing athymic nude mice over the age of 5 months old (Macdonald *et al*., 1987; Lawetzky and Hünig, 1988; Pardoll *et al*., 1988).

Macdonald and colleagues (1987) investigated TCR RNA and protein expression and identified the presence of conventional α/β TCR in nude mice lymph nodes and spleen. Additionally, through protein and DNA analysis, it was found that the T cell repertoire present in these mice is generally oligoclonal and varies between individual mice. Control *nu/+* mice did not display the same monoclonal rearrangements. A previous study carried out by the same group also noted that proportion of T cells emerging with age varies depending on genetic background, with the N:NIH II strain displaying the highest levels (MacDonald, 1984).

Lawetzky and Hunig also investigated the expression of Thy-1, CD4, CD8, $V_{\beta}8$ and CD3 in spleen and lymph node T cells of six- to twelve-month-old *nu/nu* mice on a BALB/c background (Lawetzky and Hünig, 1988). These mice contained more SP8 than SP4 T cells and these cells expressed $V_{\beta}8$ at normal proportions. However, T cells from the athymic mice expressed a lower density of $V_{\beta}8$ and CD3 than euthymic controls. To test functionality, T cells were stimulated *in vitro* with concavalin A and an anti-$V_{\beta}8$ monoclonal antibody and the proliferation response

was measured. Nude mouse T cells underwent proliferation but at four to ten-fold lower levels than thymic derived T cells, suggesting decreased functionality.

T cell accumulation with age in nude athymic mice is not however limited to the αβ T cell lineage but includes the γδ lineage. Pardoll *et al*., (1988) specifically investigated T cell subsets in the spleen of young and old *nude* mice and found that no γδ T cells are present in the young mice, but these were present in older mice alongside αβ T cells.

Kennedy and colleagues also reported the accumulation with age of T cells expressing Thy1, CD3, CD4 and CD8 in nude mice (Kennedy, Pierce and Lake, 1992). Increasing numbers of $CD4^+$ and $CD8^+$ cells were associated with increased $CD3^+$ expression over time. These authors also reported a decrease, with age, in expression of the immature T cell marker CD24, suggesting the emergence of increasing numbers of 'mature' T cells. Through flow cytometric analysis of these markers in lymph nodes and spleens of these mice, Kennedy identified the presence of immature, semi-mature and mature T cells. They also reported an inverted CD4:CD8 ratio with T cells predominantly belonging to the SP8 lineage. From the SP8 ($CD3^+CD8^+$) T cells present the majority were reported to be mature ($CD24^-$), in comparison to only half of SP4 ($CD3^+CD4^+$) T cells. When comparing lymph nodes and spleen Kennedy and colleagues also noted that regardless of the age of the animals, lymph nodes had a larger proportion of mature T cells. Macdonald and colleagues (1986) had also reported SP4 deficiency in athymic nude mice (MacDonald *et al*., 1986).

Intraepithelial lymphocytes (IEL) generated in the gut mucosa predominantly occupy the γδ over the αβ T lineage (Guy-Grand *et al*., 2003). Guy-Grand and colleagues carried out a study investigating the location of extrathymic T cell development with GFP reporter mice under the control of RAG2. Sites expressing high levels of GFP would indicate initiation or undergoing VDJ recombination events, whereas low levels would be indicative of GFP degradation taking place and therefore where gene rearrangement occurred recently. It is of note that recent thymic emigrants (RTEs) can also still express GFP due to presence of GFP even after RAG2 expression in terminated. Comparison of 18-day-old to 7-month-old *nude* mice GFP expression levels in mesenteric lymph nodes (MLN), peripheral lymph nodes, gut mucosa Peyer's patches (PPs) and cryptopatches, gut epithelium, liver, bone

marrow, lung, and peritoneal cavity was carried out. It was found that extrathymic T cell development took place in MLN and PP in the small intestine.

Palacios and Samaridis (1991) on the other hand were the first to identify the spleen as one of the extrathymic sites where TCRγ and TCRδ but not TCRβ gene rearrangement takes place in C57BL/6 *nude* mice. Although rearrangement can take place in the spleen of *nude* mice the TCR of these cells were found to be less diverse than those occurring in the thymus as identified by Southern blot analysis (Palacios and Samaridis, 1991).

All the above indicates the presence of extrathymically derived T cells that are capable of undergoing TCR gene rearrangement although with a less diverse repertoire, in relation to frequency and number of rearrangements, than thymic derived T cells. These findings are in line with the conclusions of this Chapter regarding the T cells found in the negative control Cre mice, as discussed below.

6.5.A.II. iTEC-derived peripheral T cells in the *Foxn1*$^{G/G}$ nude host

In this Chapter, I first determined the peripheral T cell development profile in *nude* mice transplanted with RFTOC, iTEC-RTOC, and Cre MEFs only controls, over a time-period of sixteen weeks post transplantation. Overall, these data showed that the major T cell subsets present in the peripheral blood of recipient mice at four weeks post grafting, did not statistically significantly vary between the Cre MEF and iTEC-RTOC conditions. However, mean absolute cell counts of all T cell subsets examined were higher in iTEC than Cre MEF recipient mice (except γδ T cells), and a trend of cell numbers increasing over time was observed. Thus, despite the lack of statistical significance (apart from αβ T cells, SP4 and antigen experienced T cells at sixteen weeks) which reflected the high degree of variability between experiments, a biological effect cannot be excluded.

By the sixteen-week timepoint, the iTEC-RTOC recipients contained a statistically significant higher mean absolute cell count of αβ T cells, SP4s and antigen experienced T cells present than the Cre MEF recipients. All other mean absolute T cell subset counts (except γδ T cells) were also increased in the iTEC-RTOC- compared to the Cre MEF- recipients at the sixteen-week timepoint, but these increases were not statistically significant. These data suggested that the iTEC-RTOC recipients had sustained T cell production for some or all of the sixteen-week time period, allowing T cell accumulation in the periphery.

Although some increase of peripheral T cell numbers was observed in the negative control recipients, this mostly reflected an accumulation of SP8s and γδ T cells, in line with studies mentioned previously. Although the host has a nude phenotype and thus lacks a thymus able to carry out thymopoiesis, *nude* mice are known to develop extrathymically derived T cells over time as discussed previously. As most mice were over the age of eight months old at the experimental endpoint, the T cells found in mice are therefore unlikely to be Cre MEF graft derived, as they do not support thymopoiesis as shown in previous Chapters. However, no age-matched controls were available to compare against for this set of experiments. Instead, T cells found in this athymic mouse model are thought to arise extrathymically similar to other nude mouse models.

When examining T cell subsets present in lymph nodes at the experimental endpoints, the iTEC-RTOC recipients also had higher mean absolute cell count for every T cell subset than the Cre MEF recipients. From all the T cell subsets however, αβ T cells, SP4s, regulatory, effector and antigen experienced T cells were significantly upregulated in comparison to the Cre recipients. The same trend was observed in spleen, although only effector and antigen experienced T cell numbers were significantly higher than in the Cre MEF condition.

Similar to the peripheral blood analysis, although there was a noticeable trend towards increased mean SP4 numbers in iTEC versus Cre MEF conditions in the lymph nodes (1.6×10^5 and 2.7×10^4) and spleen (1.4×10^5 and 1.4×10^4), the difference in mean SP8 T cell number was less profound. Instead SP8 levels found in the spleen and lymph nodes were similar in iTEC and Cre MEF recipients (spleen: 7.6×10^4 and 5.2×10^4 / lymph nodes: 1.2×10^5 and 7.8×10^4). Despite the smaller gap in SP8 numbers between the two conditions, iTEC recipients displayed higher SP8 T cell numbers than Cre MEF recipients. Thus, for this set of experiments and as expected when using *nude* recipients, SP4 T cell numbers provided the clearest evidence of thymopoiesis within the iTEC-RTOC grafts.

Additionally, Cre MEF recipients have an SP4:SP8 ratio of 0.35, iTEC-RTOC recipients of 1.28 and RFTOC recipients of 0.74 in the lymph nodes. For the spleen however, the SP4:SP8 ratio in Cre MEFs recipients is 0.26, in iTEC is 1.8 and in RFTOC is 1.27. It has been reported that an inverted (<1) CD4/CD8 ratio is associated with immunodeficiency and thymic involution due to the dwindling of the SP4 compartment (Fahey *et al*., 2000; Izaks *et al*., 2003; Akbar and Fletcher, 2005;

Shao *et al*., 2017). The CD4/CD8 ratio in iTEC recipients in the spleen and lymph nodes thus suggests rescue of immunodeficiency by repopulation of the hosts with peripheral T cells.

6.5.B. iTEC-RTOC can generate a diverse T cell repertoire

In healthy neonatal and young adult mice, peripheral T cell numbers are regulated by thymic emigration of T cells and by homeostatic mechanisms (proliferation and apoptosis) that limit overall T cell number. Studies investigating transplantation of recipients with an additional thymus however, found that the peripheral lymphocyte pool remains constant despite the presence of two fully functional thymi, suggesting thymic output isn't the only factor regulating this (Berzins, Boyd and Miller, 1998). Physical 'space' available for the lymphocytes in secondary lymphoid organs also regulate peripheral T cell numbers (reviewed in Jameson, 2002). Following thymic involution and the decline in new T cell output, however, peripheral T cell numbers are maintained via homeostatic mechanisms dynamically regulating already existing peripheral T cells (Jamieson *et al*., 1999). The process of homeostatic proliferation takes place in the spleen and lymph nodes and these locations are a requirement for T cell homeostatic proliferation to take place, as this is not observed in mutant mice that lack these environments (Dummer *et al*., 2001; Chalasani *et al.,* 2002).

It has been found that extensive homeostatic proliferation takes place during lymphopenia, regardless of the cause, such as in irradiated hosts, and in athymic (*nude*), severe combined immunodeficient (SCID) and RAG null immunodeficient mice (Mackall *et al*., 1996; Zimmermann *et al*., 1996; Ernst *et al*., 1999; Goldrath and Bevan, 1999; Kieper and Jameson, 1999; Freitas and Rocha, 2000; Kieper *et al*., 2005). Therefore, it is well documented that lymphopenic environments lead to clonal expansion in the absence of antigen stimulation, and increased T cell survival. In contrast, T cell transfer into WT animals does not result in extensive homeostatic proliferation, although if T cells are transferred into mice containing normal T cell numbers but a restricted TCR repertoire such as TCR transgenic mice, it does occur (Troy and Shen, 2003). Thus, competition between T cell clones comprises a central part of homeostatic regulation. This suggests that homeostatic proliferation is regulated by space provided in secondary lymphoid organs. IL-7 cytokine availability in these environments plays a role in facilitating this proliferation (Kieper *et al*., 2005).

Balance between thymic output, homeostatic proliferation, apoptosis and antigen specific proliferation regulates the diversity and hence functionality of the repertoire present, with antigen-specific clonal expansion taking place during infection. During infection, T cell clones specific for appropriate cognate antigens proliferate extensively, followed by deletion of majority of these antigen-specific T cells at the end of the immune response, with the aim of returning T cell numbers back to basal state (Webb, Morris and Sprent, 1990; van Parijs and Abbas, 1998).

It is important to note that homeostatic proliferation in *Foxn1*$^{G/G}$ lymphopenic hosts is likely to contribute to the kinetics of accumulation and overall numbers of T cells in the secondary lymphoid organs of the examined animals.

Having established that iTEC-RTOC rescue the T cell immunodeficiency of *Foxn1*$^{G/G}$ hosts by repopulation with peripheral T cells, I then tested the diversity of the T cell repertoires produced, as another fundamental measure of effective immune reconstitution. This also provided insight into level of clonal expansion and homeostatic proliferation taking place due to the lymphopenic environment within each recipient. All of the positive control RFTOC samples had a wide range of TRBV clones, with no clonotypes being overrepresented, indicating TCR diversity. The *Foxn1*$^{G/G}$ mice grafted with Cre MEF-MultiRTOC had a very similar profile to that reported by Macdonald *et al.*, (1987) for nude mice, namely an oligoclonal repertoire that displayed large variation in proportion of clones present between individuals.

Two iTEC recipients (ID 494, 495) displayed a diverse T cell repertoire similar to that of positive control RFTOC recipients. Thus iTEC-MultiRTOC, in some instances, are able to establish a diverse TCR repertoire similar to that of native thymic tissue transplantation.

However, some iTEC recipient animals (ID 315, 455, 501, 350, 321) exhibited high numbers of peripheral T cells but clustered with negative control Cre MEF recipients upon examination of Vβ usage, likely suggesting homeostatic proliferation of a few clones. Among these mice 455 and 350 displayed a profile similar to that of negative control Cre MEF recipients with few clones occupying a large frequency of the TCR repertoire, suggesting failure of establishment of a diverse repertoire. iTEC-RTOC recipient 321 however, displayed an intermediate diversity when compared to the RFTOC and Cre MEF recipient conditions. Mice 315 and 501 were those that had to be culled early due to rapid weight loss, a possible indicator of autoimmunity. Indeed, in each of these mice a specific T cell clone occupied up to 25% of the T cell

repertoire, suggesting homeostatic expansion of an autoreactive T cell clone. In the future, histological examination of organs for immune infiltrates and detection of autoantibodies from mouse serum would need to be carried out to determine autoimmune status of the animals examined. Although diversity in iTEC recipients that cluster with the Cre MEF condition animals appear limited, it cannot be excluded that this may be due to poor library preparation rather than true lack of TCR diversity present (as indicated by low UMI counts). A repeat experiment where all samples are standardised and prepared from the same number of T cells would help eliminate such bias. However, given the inherent variability found in the iTEC system throughout Chapter 3, 4 and 5 it is not surprising that some iTEC grafts would fail.

6.5.C. iTEC-RTOC grafts have a limited lifespan *in vivo*

In this Chapter, I also determined the longevity of iTEC-RTOC when transplanted *in vivo*. As presented in section 6.4, grafts recovered upward of seventeen weeks did not display similar architecture to that of RFTOC grafts. Instead, they closely resembled the structure of Cre MEF grafts, characterised by the absence of thymocytes, lack of K14 and UEA1 regions, and the presence of mesenchymal cells. Despite this, limited numbers of cTEC phenotype ($K8^+\beta5t^+$) iTEC were observed in the grafts at twenty-two weeks post grafting (the experimental endpoint), indicating limited longevity. In contrast, the recovered RFTOC grafts displayed the presence of thymocytes, and distinct cortical and medullary regions.

Up until now, the ability of iTEC to self-replenish has not been established. Although through constitutive expression of *Foxn1*, MEFs are able to differentiate into both cTEC and mTEC lineages, the intermediate stage prior to terminal differentiation has not been fully characterised. Therefore, it has not been confirmed whether some iTEC may attain self-renewal capacity. As iTEC have constitutive expression of *Foxn1* and should not be able to dynamically regulate their levels throughout lifespan it would be expected that cells would be constantly driven to a terminally differentiated state.

To determine their self-renewal capacity, and/or the presence of TEC progenitors within the iTEC population, iTEC would have to be examined in single cell transplantation experiments or using another assay of self-renewal (eg BrdU in conjunction with lineage tracing). Other methods of proliferation capacity could also be tested using bromodeoxyuridine (BrdU) incorporation experiments and cell

proliferation dyes to determine the exact number of cell divisions taking place in iTEC. Cellular barcoding technologies could also be employed to determine individual iTEC cell fates.

These findings pose the question of whether a single round of iTEC transplantation, at least using the current protocol for iTEC generation, is sufficient to repair the adaptive immune system of an immunodeficient host or whether multiple rounds of transplantations would be required.

6.5.D. Considerations regarding experimental design

The experiments described in this Chapter were influenced by the fact that mature T cells egressing from grafts entered an entirely lymphopenic host. Thus, the generated T cells were subject to homeostatic expansion in the periphery of the host. As T cells that first left the grafts would proliferate more extensively than T cells exiting the grafts at a later stage, this could lead to a bias of the T cell repertoire present in the periphery of the recipients. One way of improving experimental design would be to first repopulate the host with congenic T cells prior to iTEC-RTOC transplantation to remove the limitation of grafting into a lymphopenic host. This would improve the quality of the experiments in assisting in the measurement of iTEC-RTOC output in comparison to clonal expansion due to homeostatic proliferation. Performing the experiments in this manner was not considered initially as this is not a clinically relevant approach to iTEC transplantation and would not allow the investigation into how the system performs in a lymphopenic environment.

In this Chapter TRBV RNA-Seq was chosen over flow cytometry, with TCR Vβ-specific monoclonal antibodies, to determine the TCR diversity present in the periphery of the animals. Flow cytometry offers a fast and cheap technique to determine the heterogeneity of TCR Vβ gene family usage in the population of T cells present, and indeed has already been performed for grafted iTEC-RTOC (Bredenkamp *et al*., 2014). TCR repertoire analysis through flow cytometry offers qualitative and quantitative analysis of the TCRB variable region and can be used in conjunction with other markers. A limitation of this methodology is the availability of commercially available specific monoclonal antibodies, the limitation of parameters that can be examined simultaneously on a flow cytometer, lack of information regarding junctional diversity (such as CDR3 diversity) and the requirement to have sufficient cells in the gate to predict TCR repertoire diversity (Ciupe *et al*., 2013; Six

et al., 2013). Despite Next Generation Sequencing (NGS) of TCRBV genes allowing parallel analysis of millions of TCR sequences, its reliance on PCR library preparation can be associated with a number of errors due to amplification efficiency, polymerase errors and cDNA synthesis failure (Kebschull and Zabor, 2015). Alongside library preparation errors, sequencing errors must be considered as TCR clones can vary from each other by a single nucleotide. It is therefore important to differentiate between actual low clonotype diversity and possible PCR and sequencing errors when considering TCR diversity through this methodology. Although the two methodologies each offer advantages and disadvantages, NGS of TCRB was carried out due to its ability to identify a larger number of TCR clones and thus examine the repertoires in detail. The data generated may also contribute to baseline data in experiments that examine the response to immunological challenge in iTEC-grafted mice.

As we were aware of the technical issues associated with the protocol, UMI added during cDNA synthesis were utilised to estimate PCR amplification and sequencing errors (Kivioja *et al*., 2011). In addition, algorithms such as the molecular identifier groups-based error correction (MiGEC) tool, developed to account for these errors and give a better estimation of TCR diversity present (Bolotin *et al*., 2012), were applied.

Regarding extrathymic and graft derived T cells, for future experiments, it would be beneficial to include both α-CD8α and α-CD8β chain antibodies. Extrathymic T cells have been characterised to have CD8αα coreceptor expression, whereαs T cells that develop in the thymus express CD8αβ (Guy-Grand and Vassalli, 1991; 1993). Some studies have shown however that some extrathymically derived SP8 T cells in nude mice can express the CD8αβ heterodimer, albeit at lower proportions than CD8αα and with variability between individual mice (Emoto, Emoto and Kaufmann).

6.6. Conclusions

To conclude, in this Chapter I present evidence that iTEC-RTOC repopulate the periphery of *Foxn1*$^{G/G}$ T cell immunodeficient hosts and have the ability to generate a diverse TCR repertoire. In addition, although grafts with typical thymus histology are present at early timepoints, histological examination has concluded that iTEC grafts transplanted for more than seventeen weeks *in vivo* showcase very few recovered cTEC, with no mTEC present, indicating the limited longevity of iTEC grafts.

These findings are important for the goal of establishing iTEC as a tool for repairing the adaptive immune system and furthering the development of this system for a future clinical setting.