Supplemental Material

Intratumoral T-cells have a Differential Impact on FDG-PET Parameters in Follicular Lymphoma

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FDG-PET parameters

Pre-treatment ^{18}F-fluorodeoxyglucose-positron emission tomography-computed tomography (FDG-PET) data in digital imaging and communications (DICOM) format were obtained for functional parameter measurements. All FDG-PET scans were obtained from the same scanner.

Two independent reviewers computed maximum standardized uptake value (SUV_{max}), total metabolic tumor volume (TMTV) and total lesional glycolysis (TLG) using a customized MIM software workflow (MIM Software Inc, OH, USA). Maximum standardized uptake value (SUV_{max}) was defined as the single voxel with the highest uptake in a lesion. Individual lesions of interest were identified by visual assessment of FDG-PET images and thereafter TMTV was obtained by summing the metabolic volume of all lesions. TMTV was calculated using a (i) SUV ≥41% of lesional SUV_{max} method (41%SUV_{max}) as recommended by the European Association of Nuclear Medicine,[1] and (2) fixed SUV ≥2.5 threshold method (SUV≥2.5), as previously described.[2,3] TLG is the sum of the MTVs (using 41% SUV_{max} method) of individual lesions multiplied by their average SUV.[1] The bone marrow was considered involved only if there was focal uptake, and the spleen was included if there was either focal uptake or diffuse uptake 150% higher than the background liver activity.[4]

Twelve patients had a pre-therapy FDG-PET scan performed *prior* to their diagnostic excisional node biopsy. Lesional SUV_{max} ($SUV_{lesional}$) within the subsequently excised node was obtained, providing a direct measure of FDG avidity within the tissue biopsy. In these 12 patients, the median time interval between the FDG-PET scan and subsequent biopsy was 15 days (interquartile range, 5-38 days). And in the patients who had their excisional biopsy prior to their FDG-PET scan (n=51), the median time interval between the biopsy and scan was 20 days (interquartile range, 8-29 days).

Gene expression

In the Brisbane cohort, RNA was extracted from sequential scrolls of archival follicular lymphoma (FL) formalin-fixed paraffin embedded (FFPE) samples using the Qiagen AllPrep DNA/RNA FFPE Kit. T-cell immune infiltration was determined using a targeted NanoString (NanoString Technologies, USA) panel of 12 clinically pertinent immune effector (*CD137*, *CD4*, *CD7*, *CD8A*, *TNFα*), immune checkpoint (*PD-1*, *PD-L1*, *PD-L2*, *TIM3*, *LAG3*, *FOXP3*)

and macrophage (*CD68*) molecules, as previously published.[5] Normalization genes used in this code-set were *GAPDH*, *OAZ1*, *PGAM1* and *PGK1*. For each sample, a standardized Z-score was computed from *CD4* and *CD8A* gene counts. The average *CD4*, *CD8A* Z-score was then used to categorize T-cell^{LO} (quartile 1-3) and T-cell^{RICH} (quartile 4) nodes. Normalized data is provided in **supplemental Table S3**.

In the twelve patients in whom a staging FDG-PET scan was performed prior to their diagnostic excisional node biopsy, the Maxwell RNA FFPE Kit was used for RNA extraction. The nCounter PanCancer immune profiling panel (730 immune related genes + 40 housekeeping genes, NanoString) was used to permit analysis of *CD4*, *CD8A*, *CD19* and *CD68* gene expression within the subsequently biopsied FL node. This allowed to test for an association between the FDG-PET derived $SUV_{lesional}$ status and *CD4*, *CD8A*, *CD19* and *CD68* gene expression from the same lesion. Normalized data is provided in the **supplemental Table S4**.

TCRβ sequencing

Twenty-one fresh de-aggregated and immediately cryopreserved baseline FL tumor infiltrating lymphocytes (TILs) from the Brisbane cohort were used. Samples underwent fluorescence activated cell sorting (FACS) into phenotypically distinct intra-tumoral T-cell subsets: T-follicular helper cells (T_{FH} cells; $CXCR5^+ICOS^+CD4^+$), regulatory T-cells (T_{REG}; $CD25^{HI}CD127^{LO}CXCR5^{-ve}CD4^+$), non-$T_{FH}$/$T_{REG}$ $CD4^+$ T-cells and $CD8^+$ T-cells.[6] Of the 21 FL TILs, 14 underwent further $CD8^+$ cell sorting into distinct PD-1^+LAG^{-ve} (activated), PD-1^+LAG3^+ (exhausted) and PD-$1^{-ve}LAG3^{-ve}$ (resting) $CD8^+$ T-cells.[7] Flow-cytometry antibodies and fluorochromes are included in **supplemental Table S5**. Total genomic DNA was then extracted within these individual T-cell subsets in each patient using the QIAamp DNA Micro Kit (Qiagen). High-throughput, deep-resolution (3-6 replicates) next-generation sequencing (NGS) of the TCRβ gene was performed using the immunoSEQ hsTCRβ assay (Adaptive Biotechnologies), as previously described.[8,9] In brief, TCRβ CDR3 regions were amplified by a multiplex, bias-controlled PCR with a pool of primer pairs targeting *V* and *J* genes of T-cells, and subsequently tagged with unique barcoded primer pairs. Purified barcoded PCR products were sequenced on an Illumina NextSeq-500 analyzer. Following quality control review, the raw NGS data were uploaded onto the immunoSEQ pipeline and Simpson's clonality was calculated according to the equation: $\sum_{i=1}^{R} P_i^2$ where R = total number of rearrangements; i = each rearrangement; P_i = productive frequency of rearrangement. The Simpson's clonality metric ranges from 0 to 1 where values approaching 0 represent a completely even sample, and 1 represents a monoclonal sample. 'All $CD4^+$' T-cell clonality was generated by merging

the individual DNA rearrangements from the 3 $CD4^+$ T-cells subsets, and subsequently calculating clonality.

T-cell clonality values within all sorted T-cell subsets was independent to, and not associated with the $CD19^+$ B-cell percentage within malignant nodes (as enumerated by flow cytometry).

Analysis of cellular glucose-uptake

Eight FL TILs from the Brisbane cohort and 20 FL TILs from the Canberra cohort were used to assess cellular glucose-uptake within $CD4^+$ T-cells, $CD8^+$ T-cells and $CD19^+$ B-cells. Dimethyl sulphoxide (DMSO) was used as a cryoprotective agent to maintain cell viability of TILs.

The 2-NBDG glucose-uptake assay kit (Abcam) was used to determine cellular glucose-uptake.[10] The kit uses 2-NBDG, a fluorescently labelled deoxyglucose analogue. FL TILs were rapidly thawed and assayed for viability and a minimum of 200,000 live cells were required. Thereafter, cells were washed, resuspended in complete cell-growth medium (RPMI1640 + 1% sodium pyruvate + 1% penicillin-streptomycin-glutamine + 10% heat-inactivated FBS, Gibco) and rested at 37°C for 2 hours. Cells were then washed and rested glucose free RPMI1640 medium (Gibco) for 2 hours. After 2 hours of glucose starvation, 12.5µg/mL 2-NBDG was added to cells and incubated at 37°C for 1 hour, as determined by our in-house optimisation assay. After additional washes, cells were incubated with the surface antibody cocktail following manufacturer's instruction. The samples were analysed on FACSymphony A5 and data was analysed using Kaluza software (Beckman Coulter). Flow-cytometry antibodies and fluorochromes are included in **supplemental Table S5**.

Histopathology and flow cytometry of diagnostic tissue biopsies

FL diagnostic histopathology reports (from excisional lymph node biopsies), which includes the FL histological grading, were obtained. Where available, H&E-stained tissue sections for Ki67 immunohistochemistry (IHC) were reviewed from the Brisbane cohort. In the 33 diagnostic biopsies that had Ki67 IHC, these were separated into a low Ki67 (<20%) and high Ki67 (≥20%) expression, as previously published.[11] Additionally, paired diagnostic tissue flow cytometry reports from the Brisbane cohort were obtained to assess infiltration by light-chain (kappa or lambda) restricted $CD19^+$ FL B-cells.[12] Flow cytometric analysis was performed on BDFACS Canto (BD Biosciences).

Statistical Analysis

Comparisons of patient variables between groups used the Wilcoxon rank-sum test. *P* values <0.05 were statistically significant. Statistical analysis used Prism v7.03 (GraphPad Software, CA).

Supplementary References

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Supplemental Table S1. Correlative analysis between $CD4^+$, $CD8^+$ and $CD19^+$ cellular glucose uptake and $SUV_{lesional}$.

CELL SUBSET	Pearson r	*P*-value
$CD4^+$	0.70	0.054
$CD8^+$	0.44	0.280
$CD19^+$	0.55	0.156

Supplemental Table S2. Baseline pre-therapy FDG-PET parameters

FL No.	TMTV, cm^3 (41%SUV_{max})	TMTV, cm^3 (SUV≥ 2.5)	Highest SUV_{max}	TLG, g
1	451.03	680.56	23.44	5384.28
2	14.41	14.47	6.25	63.48
3	18.69	33.33	23.23	140.98
4	29.43	43.47	6.86	37.84
5	247.73	282.26	12.07	1616.95
6	32.31	33.06	3.22	64.16
7	602.68	1731.89	37.36	10107.12
8	2518.82	2691.83	7.41	8033.26
9	701.46	1108.4	8.98	2485.95
10	942.04	1080.66	11.1	4711.43
11	268.35	484.98	21.62	3569.1
12	824.02	1724.09	7.7	3539.68
13	108.74	111.07	6.11	252.8
14	384.33	485.68	11.45	1339.22
15	619.69	1636.85	16.26	5155.21
16	21.87	33.67	13	165.85
17	65.44	83.5	9.06	312.11
18	627.42	748.94	8.59	2906.68
19	73.07	154	9.76	293.13
20	262.22	943.63	15.89	2069.32
21	10.04	12	7.31	42.15
22	166.87	1437.59	23.4	1280.65
23	1810.78	2297.94	8.18	7424.03
24	1097.8	1603.98	10.27	5863.26
25	283.15	575.97	20.41	2550.06
26	142.55	148.66	4.2	242.7
27	465.46	1740.19	14.46	1553.06
28	42.98	51.57	14.81	90.96
29	18.58	19.23	4.14	38.01
30	86.52	101.37	11.63	346.97
31	59.99	70.92	5.14	146.49
32	6.57	6.57	3.84	20.92
33	487.78	747.33	9.47	2292.45
34	110.39	127.84	7.98	449.94
35	1465.85	1841.61	25.72	7583.15
36	218.57	266.84	7.9	1034.26
37	430.24	523.01	5.66	1298.15
38	1830.67	3172.58	11.3	9005.98
39	1642.9	3426.23	10.54	8712.75
40	232.11	1130.26	18.14	1799.4

FL No.	TMTV, cm^3 (41%SUV_{max})	TMTV, cm^3 (SUV≥ 2.5)	Highest SUV_{max}	TLG, g
41	29.61	38	8.97	169.54
42	75.51	79.69	6.02	272.88
43	16.3	24.25	8.09	75.6
44	16.39	18.50	7.39	70.93
45	208.78	277.12	5.93	609.04
46	154.27	436.72	9.53	483.22
47	17.47	21.97	6.49	26.91
48	23.16	25.42	4.93	62.01
49	2527.58	4442.49	10.67	11319.99
50	994.82	1476.06	9.6	2878.34
51	25.88	26.12	6.65	59.74
52	32.66	34.73	8.45	146.27
53	11.64	13.5	4.33	29.1
54	650.75	1550.72	25.39	5028.63
55	98.14	115.22	6.61	242.12
56	44.62	46.99	5.89	139.31
57	48.19	78.01	7.93	209.8
58	751.68	1531.51	17.29	4118.98
59	268.29	402.52	8.71	1040.16
60	709.38	921.97	10.18	2715.33
61	295.16	825.55	13.49	1683.79
62	1416.92	2409.98	8.13	4848.69
63	447.21	886.68	9.36	1585.87

Abbreviations: TMTV, total metabolic tumor volume; SUV_{max}, maximum standardized uptake value; TLG, total lesion glycolysis

Supplemental Table S3. Normalized *CD4* and *CD8A* gene expression in follicular lymphoma patients

FL No.	Age	Sex	Stage	FLIPI	*CD4*	*CD8A*
1	49.8	M	4	2	1528.26	1582.12
2	72.4	M	1	2	3472.18	1134.68
3	77.4	M	1	1	1579.91	1297.89
4	51.7	F	3	2	3281.32	1701.83
5	58.2	F	4	3	2152.18	2654.36
6	45.9	M	1	2	2122.14	1055.99
7	45.6	F	4	3	2273.92	2052.49
8	69.2	M	4	3	1149.73	619.84
9	73.9	F	3	3	560.4	311.44
10	30.5	F	4	3	1233.21	1706.84
11	46.2	F	2	2	1589.21	1412.41
12	56.1	M	4	1	2189.49	2137.71
13	71.1	M	4	3	3600.68	7135.29
14	68.8	F	4	3	186.65	191.7
15	64.0	F	4	3	1321.24	3312.78
16	56.6	F	4	2	1726.45	1915.33
17	67.1	M	4	3	2200.14	3433.24
18	49.8	F	4	2	1614.59	1606.15
19	60.9	F	3	3	2127.28	2279.96
20	83.4	F	3	3	2022.32	2135.45
21	56.0	M	2	1	775.54	895.75
22	29.2	M	3	1	1922.19	1201.93
23	44.9	M	4	3	2474.09	1201.41
24	58.4	F	3	1	165.89	180.97
25	54.5	F	3	3	1630.82	1444.77
26	51.3	F	4	3	1388.36	1227.68
27	60.2	F	3	3	507.79	546.5
28	71.9	F	2	1	3627.21	3990.09
29	50.6	F	3	2	2436.68	3897.52
30	68.4	F	3	3	816.74	533.64
31	43.0	F	2	1	2583.63	7567
32	83.5	F	1	1	822.56	1821.76
33	52.9	F	4	3	1492.58	4265.08
34	52.5	F	4	2	2390.24	3145.72
35	56.9	M	4	2	1304.4	1576.55
36	62.9	M	4	3	1944.46	864.16
37	69.6	F	4	3	3347.28	493.08
38	65.4	M	4	3	955.84	1106.61
39	52.4	M	4	2	2668.71	1093.49
40	53.2	F	4	2	1541.19	1076.71

FL No.	Age	Sex	Stage	FLIPI	*CD4*	*CD8A*
41	61.0	M	3	2	2527.18	854.12
42	81.7	F	4	3	3385.49	1698.71
43	63.3	M	3	2	3067.06	1086.67
44	68.8	F	3	2	3368.61	601.2
45	76.9	M	3	3	2670.34	6226.68

Supplemental Table S4. Normalized *CD4*, *CD8A*, *CD19* and *CD68* gene expression and $SUV_{lesional}$ values from pre-biopsy FDG-PET scans

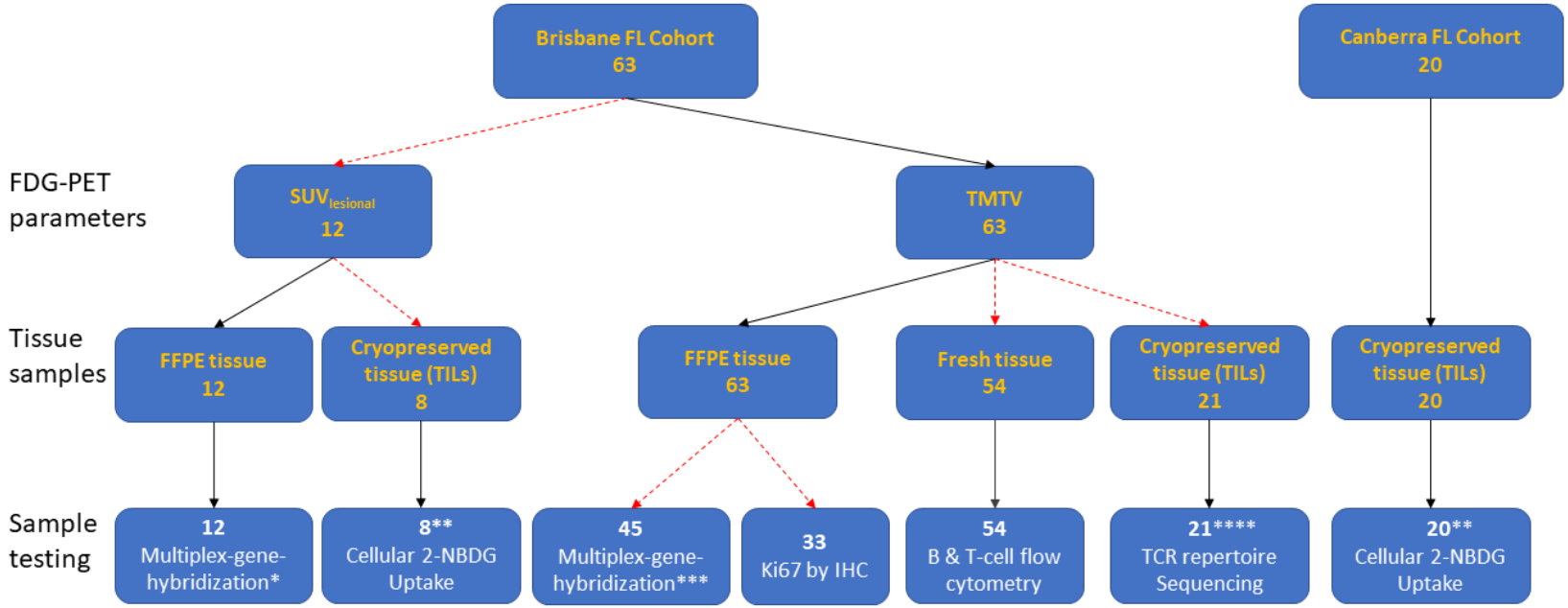
FL No.	Age	Sex	Stage	FLIPI	*CD4*	*CD8A*	*CD68*	*CD19*	$SUV_{lesional}$
14	69	F	4	3	298.48	85.77	253.88	20	3.54
21	56	M	2	1	838.91	513.01	421.31	2643.12	6.53
25	55	F	3	3	751.4	296.15	357.58	3367.9	8.15
48	72	F	4	2	361.52	241.93	333.92	982.45	4.78
51	40	F	3	2	433.44	495.14	277.65	1261.77	6.65
52	70	F	1	1	777.70	672.51	451.49	696.15	7.54
53	78	F	2	2	360.22	321.57	234.22	779.20	4.33
55	72	F	3	2	569.49	229.04	578.86	1154.59	5.24
56	40	M	4	2	358.82	212.63	242.76	759.28	5.89
57	71	M	4	3	696.36	653.82	709.57	1009.61	7.93
58	67	F	4	5	665.79	900.71	455.23	741.82	7.97
60	75	M	4	2	465.96	335.8	436.43	962.55	8.99

Abbreviations: $SUV_{lesional}$, lesional SUV_{max}

Supplemental Table S5. Flow-cytometry antibodies and fluorochromes used for fluorescence activated cell sorting (FACS) sorting of intratumoral T-cell subsets for TCRβ sequencing, and for analysis of cellular glucose-uptake

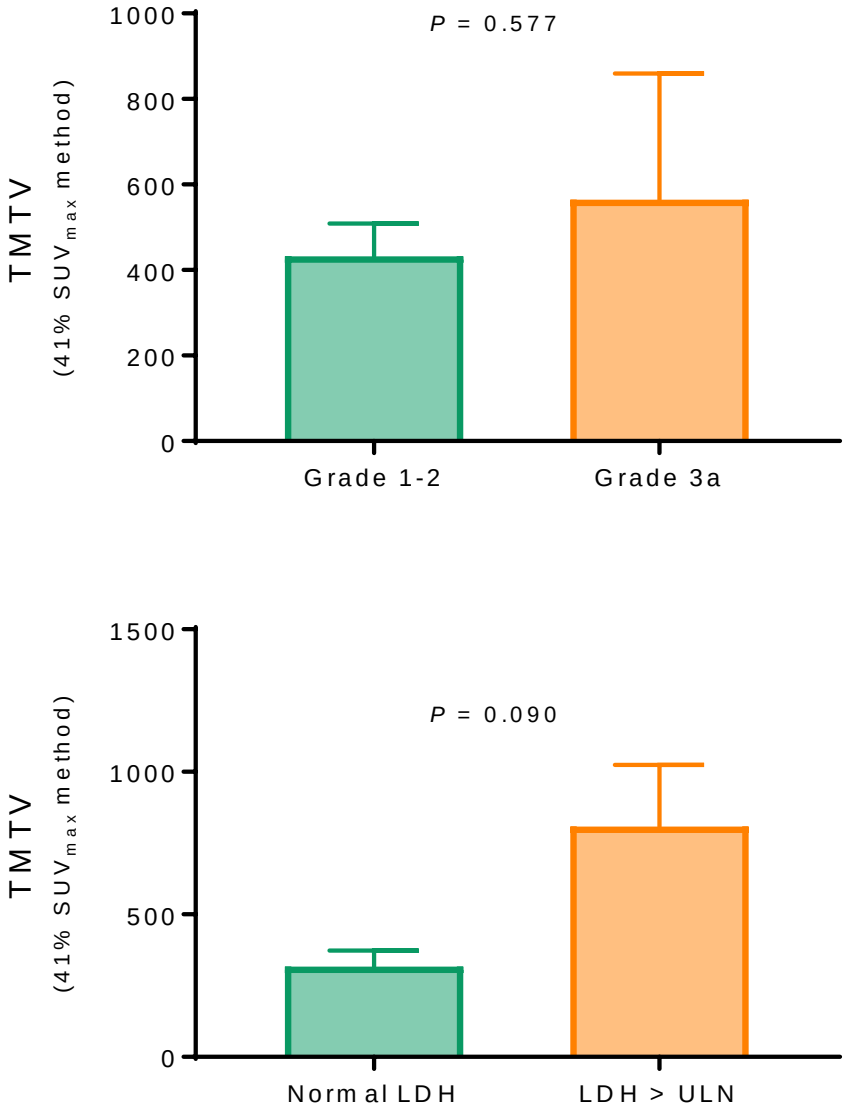
Marker	Fluorochrome	Clone	Isotype	Manufacturer
Different T-cell subsets panel				
CD11c	FITC	B-ly6	Mouse IgG1, κ	BD Biosciences
CD14	FITC	MϕP9	Mouse IgG2b, κ	BD Biosciences
CD16	FITC	3G8	Mouse IgG1, κ	Biolegend
CD19	FITC	HIB19	Mouse IgG1, κ	BD Biosciences
ICOS	PE	DX29	Mouse IgG1, κ	BD Biosciences
CD127	PerCP/Cy5.5	hIL-7R-M21	Mouse IgG1, κ	BD Biosciences
CD25	APC	BC96	Mouse IgG1, κ	Biolegend
CXCR5	BV421	J252D4	Mouse IgG1, κ	Biolegend
CD8	BV785	SK1	Mouse IgG1, κ	Biolegend
CD3	BUV395	SK7	Mouse IgG1, κ	BD Biosciences
CD4	BUV737	SK3	Mouse IgG1, κ	BD Biosciences
Live/dead fixable green dead cell stain	488 nm	-	-	Invitrogen
CD8 subsets panel				
CD11c	FITC	B-ly6	Mouse IgG1, κ	BD Biosciences
CD14	FITC	MϕP9	Mouse IgG2b, κ	BD Biosciences
CD16	FITC	3G8	Mouse IgG1, κ	Biolegend
CD19	FITC	HIB19	Mouse IgG1, κ	BD Biosciences
CD3	APC	UCHT1	Mouse IgG1, κ	BD Pharmingen
CD8	BV785	SK1	Mouse IgG1, κ	Biolegend
PD-1	PE	PD1.3.1.3	Mouse IgG2b, κ	Miltenyi Biotec
LAG3	BUV395	T45-530	Mouse IgG1, κ	BD Biosciences
Live/dead fixable green dead cell stain	488 nm	-	-	Invitrogen
2-NBDG panel				
CD11c	PE/Cy5	B-ly6	Mouse IgG1, κ	BD Biosciences
CD14	AF700	M5E2	Mouse IgG2a, κ	BD Biosciences
CD16	PE/Cy5	3G8	Mouse IgG1, κ	BD Biosciences
CD19	PE/Cy7	SJ25C1	Mouse IgG1, κ	BD Biosciences
CD3	BUV496	UCHT1	Mouse IgG1, κ	BD Biosciences
CD4	BUV805	SK3	Mouse IgG1, κ	BD Biosciences
CD8	BUV563	RPA-T8	Mouse IgG1, κ	BD Biosciences
Fixable viability stain 700	640 nm	-	-	BD Biosciences
2-NBDG	488 nm	-	-	Abcam

Supplemental Figure S1. Consort diagram providing details of FDG-PET parameters, FL *de-novo* tumor tissue and sample testing performed.



Brisbane FL Cohort from the Princess Alexandra Hospital; Canberra FL Cohort from the Canberra Hospital. *Multiplex-gene-hybridization (NanoString) was performed to permit analysis of *CD4*, *CD8A*, *CD19* and *CD68* gene expression. **8 FL TILs from the Brisbane Cohort and 20 FL TILs from the Canberra cohort underwent cellular 2-NBDG assessment within $CD19^{+}$, $CD8^{+}$ and $CD4^{+}$ cell subsets. ***Multiplex-gene-hybridization (NanoString) was performed to determine intra-tumoral T-cell states ($T\text{-}cell^{LO}$ vs. $T\text{-}cell^{RICH}$). ****Twenty-one FL TILs underwent TCR repertoire sequencing within FACS sorted $CD4^{+}$ T_{FH}, T_{REG}, Non-T_{FH}/T_{REG} cells and $CD8^{+}$ T-cells. Of these 21 FL TILs, 14 underwent further TCR repertoire sequencing within sorted $PD1^{+}LAG3^{-ve}$, $PD1^{+}LAG3^{+}$ and $PD1^{-ve}LAG3^{-ve}$ $CD8^{+}$ T-cells. Abbreviations: FL: follicular lymphoma; $SUV_{lesional}$: lesional SUV_{max}; TMTV: total metabolic tumor volume; FFPE: formalin-fixed paraffin embedded; TILs: tumor infiltrating lymphocytes; IHC: immunohistochemistry; TCR: T-cell receptor.

Supplemental Figure S2. Histological FL grade and serum lactate dehydrogenase (LDH) are not associated with pre-treatment TMTV



TMTV (cm^3) was determined by the 41%SUV_{max} method. Grade 1-2 (*n* = 55) and Grade 3 (*n* = 8); Normal LDH (*n* = 46) and LDH > upper limit of normal (ULN, *n* = 17).