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Supplemental Information

An HIF-1α/VEGF-A Axis in Cytotoxic T Cells Regulates Tumor Progression

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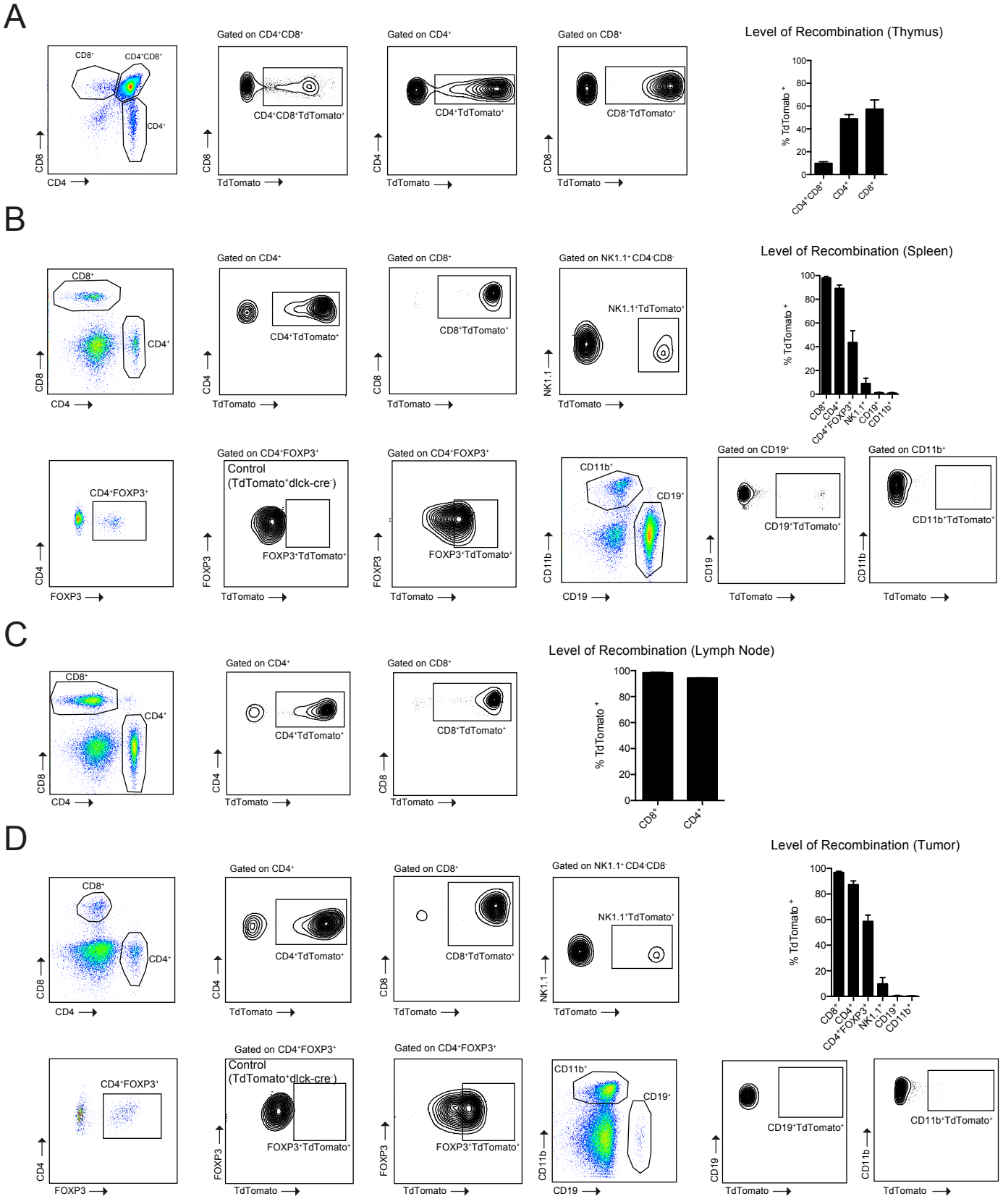


Figure S1, related to Figure 1.

(A-D) Level of dlck-cre recombination in thymus (A), spleen (B), lymph nodes (C), and subcutaneous LLC tumors (D) (n=4) was measured using a TdTomato reporter mouse line by flow cytometry. Error bars represent SD.

Table S1, related to Figure 1.

	Mean pO_2 (mmHg)	SD
Culture media 21% O_2	107.38	2.65
Culture media 5% O_2	31.65	0.26
Culture media 1% O_2	10.33	4.25
Spleen	21.73	12.6
Tumor (LLC)	1.95	2.41

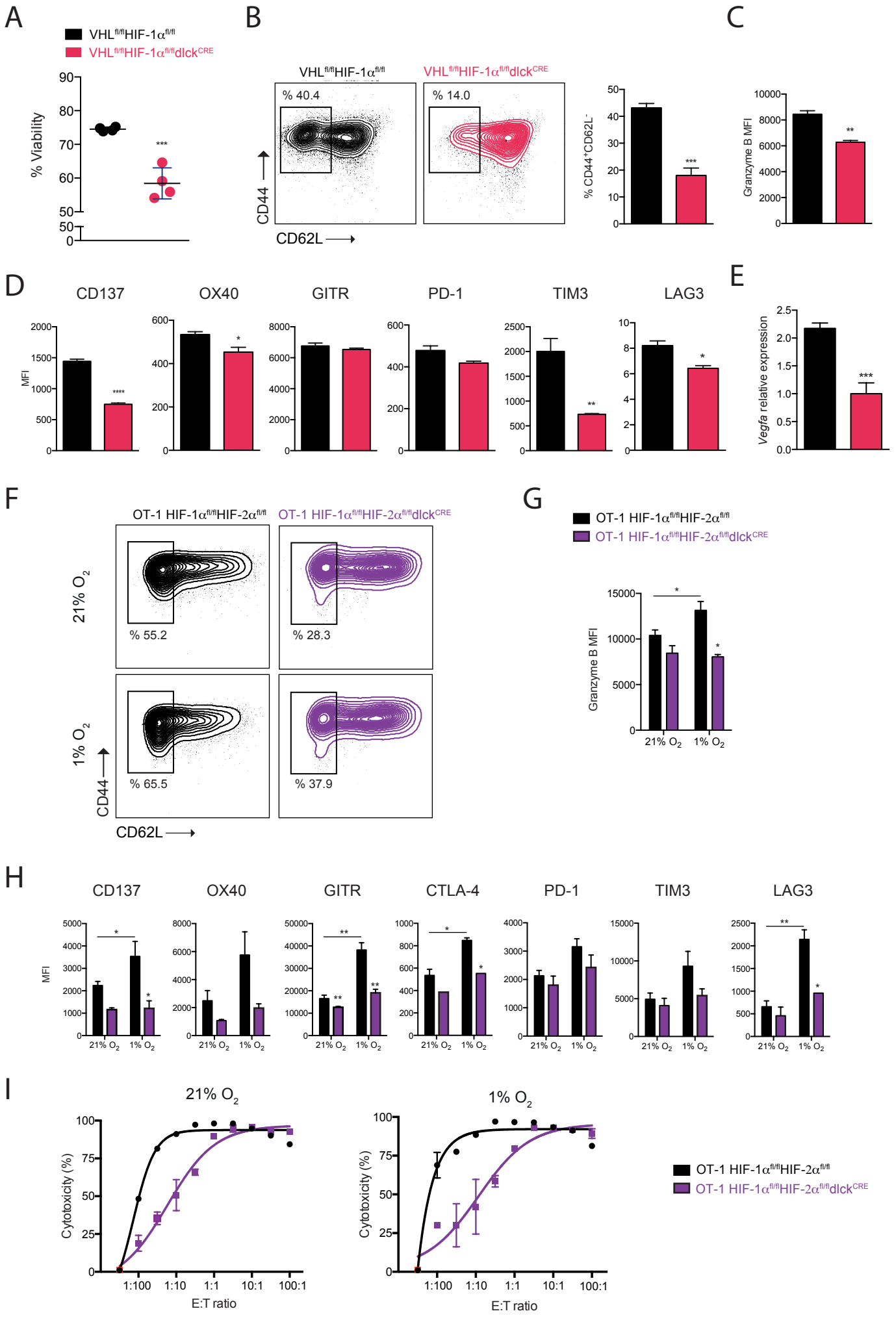

A
VHL^fl/fl HIF-1α^fl/fl
VHL^fl/fl HIF-1α^fl/fl dlck^CRE
% Viability
B
VHL^fl/fl HIF-1α^fl/fl
VHL^fl/fl HIF-1α^fl/fl dlck^CRE
% 40.4
% 14.0
CD44
CD62L
% CD44+CD62L-
C
Granzyme B MFI
D
CD137
OX40
GITR
PD-1
TIM3
LAG3
MFI
E
Vegfa relative expression
F
OT-1 HIF-1α^fl/fl HIF-2α^fl/fl
OT-1 HIF-1α^fl/fl HIF-2α^fl/fl dlck^CRE
21% O2
1% O2
% 55.2
% 28.3
% 65.5
% 37.9
CD44
CD62L
G
OT-1 HIF-1α^fl/fl HIF-2α^fl/fl
OT-1 HIF-1α^fl/fl HIF-2α^fl/fl dlck^CRE
Granzyme B MFI
21% O2
1% O2
H
CD137
OX40
GITR
CTLA-4
PD-1
TIM3
LAG3
MFI
I
21% O2
1% O2
Cytotoxicity (%)
E:T ratio
1:100
1:10
1:1
10:1
100:1
OT-1 HIF-1α^fl/fl HIF-2α^fl/fl
OT-1 HIF-1α^fl/fl HIF-2α^fl/fl dlck^CRE

Figure S2, related to Figure 2.

(A-E) $CD8^+$ T cells were isolated from spleens of $VHL^{fl/fl}HIF\text{-}1\alpha^{fl/fl}dlck^{CRE}$ (pink) and littermate control (black) mice, activated with αCD3/αCD28 for 48 hr, and then expanded for 5 days in the presence of IL-2: percent survival (A), expression of CD44 and CD62L by flow cytometry (B), expression of Granzyme B by intracellular flow cytometry (C), surface expression of costimulatory molecules/checkpoint receptors CD137, OX40, GITR, PD-1, TIM-3 and LAG3 by flow cytometry (D) and relative expression of *Vegfa* by QRT-PCR (E), n=4, error bars represent SD. (F-H) $CD8^+$ T cells were isolated from spleens of OT-1 $HIF\text{-}1\alpha^{fl/fl}HIF\text{-}2\alpha^{fl/fl}dlck^{CRE}$ (purple) and littermate control (black) mice, activated with cognate peptide for 2 days, and then expanded for 5 days in the presence of IL-2 and subjected to 21% or 1% O_2 for 24 hr: expression of CD44 and CD62L by flow cytometry (F) expression of Granzyme B by intracellular flow cytometry (G), expression of the indicated costimulatory molecules/checkpoint receptors by flow cytometry (H), n=4, error bars represent SD. (I) *In vitro* cytotoxicity assay: EG7-OVA target cells and control or mutant OT-1 $CD8^+$ T cells were co-cultured at different effector to target (E:T) ratios for 24 hr under 21% or 1% O_2 (n=2, error bars represent SE).

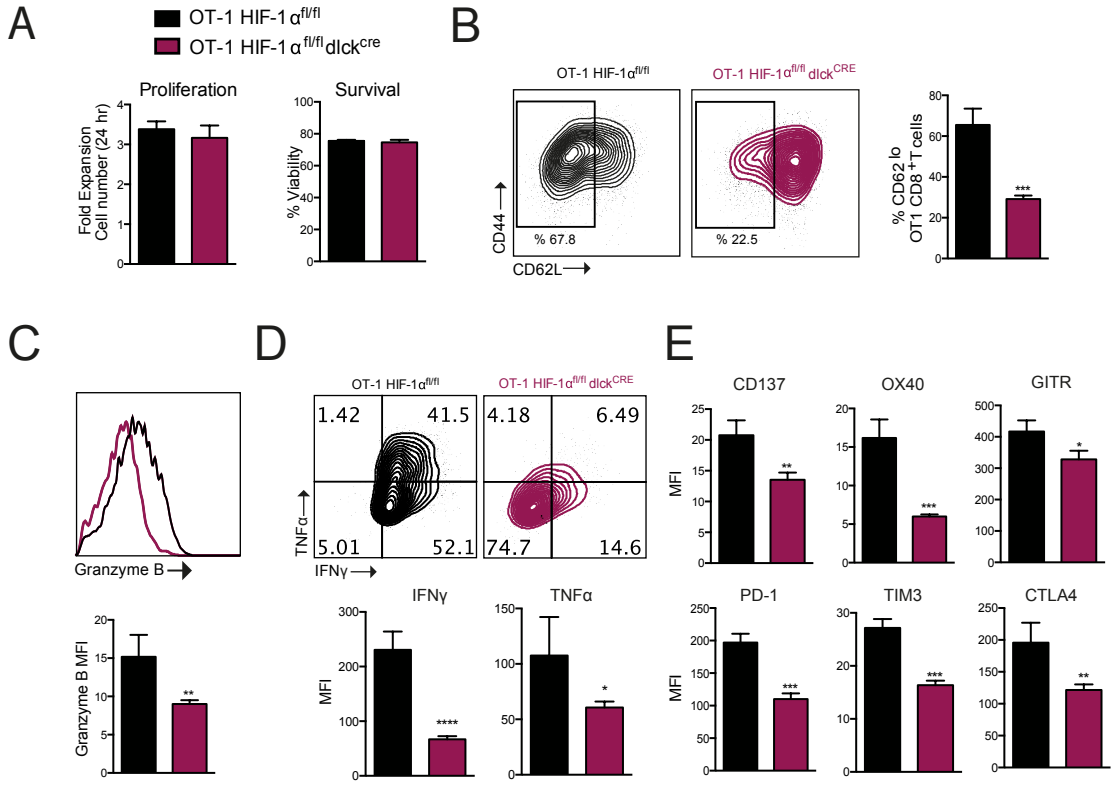
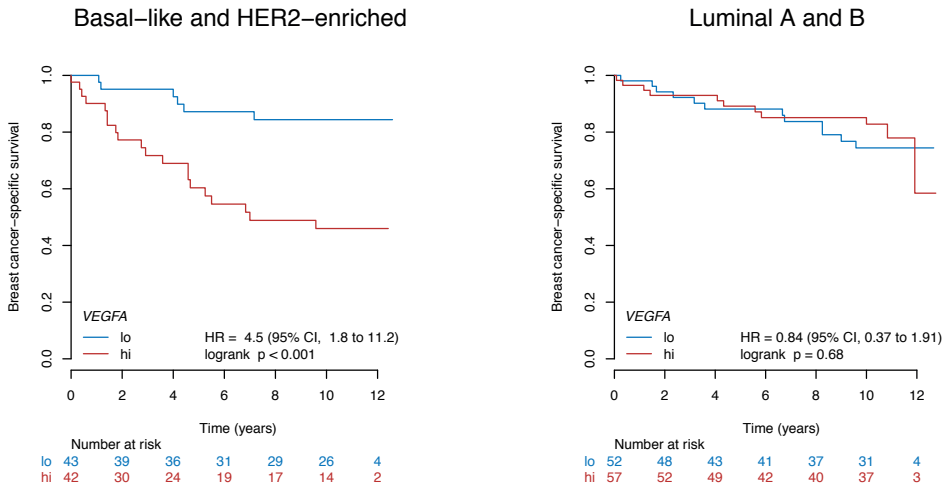

A
OT-1 HIF-1α fl/fl
OT-1 HIF-1α fl/fl dIck cre
Proliferation
Fold Expansion Cell number (24 hr)
Survival
% Viability
B
OT-1 HIF-1α fl/fl
OT-1 HIF-1α fl/fl dIck CRE
% 67.8
% 22.5
CD44
CD62L
% CD62 lo OT1 CD8 + T cells
C
Granzyme B
Granzyme B MFI
D
OT-1 HIF-1α fl/fl
OT-1 HIF-1α fl/fl dIck CRE
1.42
41.5
5.01
52.1
4.18
6.49
74.7
14.6
TNFα
IFNγ
IFNγ
TNFα
MFI
E
CD137
OX40
GITR
PD-1
TIM3
CTLA4
MFI

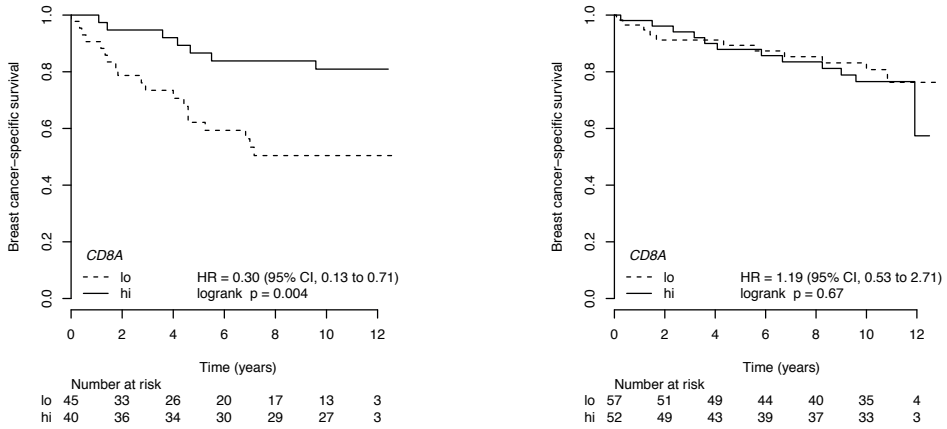
Figure S3, related to Figure 4.

(A-B) Splenic OT-1 cells were activated *in vitro* with cognate SIINFEKL peptide and expanded under 21% O_2 for 3 days in the presence of IL-2: proliferation and survival (A), surface CD62L expression by flow cytometry (B), (n=3, error bars represent SD). (C) Intracellular granzyme B expression (n=3, error bars represent SD). (D) Intracellular TNFα and IFNγ expression after 4 hr *in vitro* restimulation with SIINFEKL (n=3, error bars represent SD). (E) Surface expression of costimulatory/checkpoint receptors CD137, OX40, GITR, PD-1 and TIM3 and intracellular expression of CTLA4 (n=3, error bars represent SD). Two-tailed student T test was used for comparisons.

A


Basal−like and HER2−enriched
Luminal A and B
Breast cancer−specific survival
VEGFA
lo
hi
HR = 4.5 (95% CI, 1.8 to 11.2)
logrank p < 0.001
HR = 0.84 (95% CI, 0.37 to 1.91)
logrank p = 0.68
Time (years)
Number at risk
lo 43 39 36 31 29 26 4
hi 42 30 24 19 17 14 2
lo 52 48 43 41 37 31 4
hi 57 52 49 42 40 37 3

B


Basal−like and HER2−enriched
Luminal A and B
Breast cancer−specific survival
CD8A
lo
hi
HR = 0.30 (95% CI, 0.13 to 0.71)
logrank p = 0.004
HR = 1.19 (95% CI, 0.53 to 2.71)
logrank p = 0.67
Time (years)
Number at risk
lo 45 33 26 20 17 13 3
hi 40 36 34 30 29 27 3
lo 57 51 49 44 40 35 4
hi 52 49 43 39 37 33 3

C

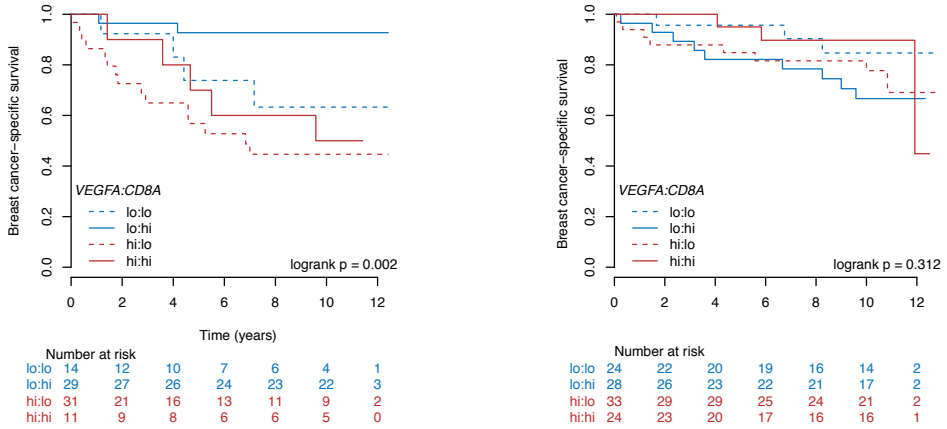

Basal−like and HER2−enriched
Luminal A and B
Breast cancer−specific survival
VEGFA:CD8A
lo:lo
lo:hi
hi:lo
hi:hi
logrank p = 0.002
logrank p = 0.312
Time (years)
Number at risk
lo:lo 14 12 10 7 6 4 1
lo:hi 29 27 26 24 23 22 3
hi:lo 31 21 16 13 11 9 2
hi:hi 11 9 8 6 6 5 0
lo:lo 24 22 20 19 16 14 2
lo:hi 28 26 23 22 21 17 2
hi:lo 33 29 29 25 24 21 2
hi:hi 24 23 20 17 16 16 1

Figure S4, related to Figure 5.
(A-C) Association of *VEGFA* and *CD8A* expression with clinical outcome in the Uppsala cohort. Kaplan-Meyer survival curves of breast-cancer specific survival are shown for the indicated subtypes, for patients classified according to *VEGFA* relative expression levels (A), *CD8A* relative expression levels (B), and different ratios of *VEGFA*:*CD8A* relative expression levels (C).

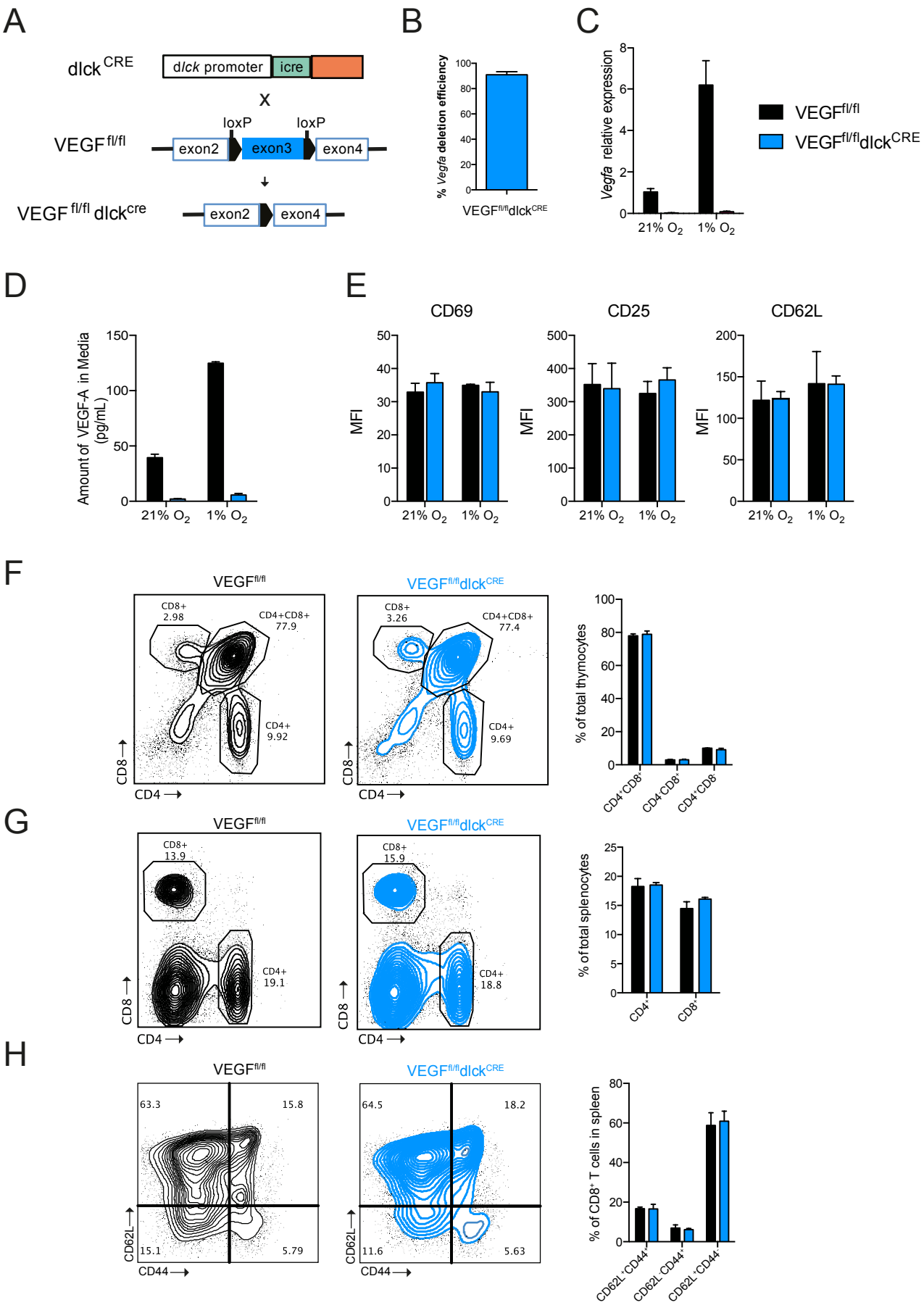
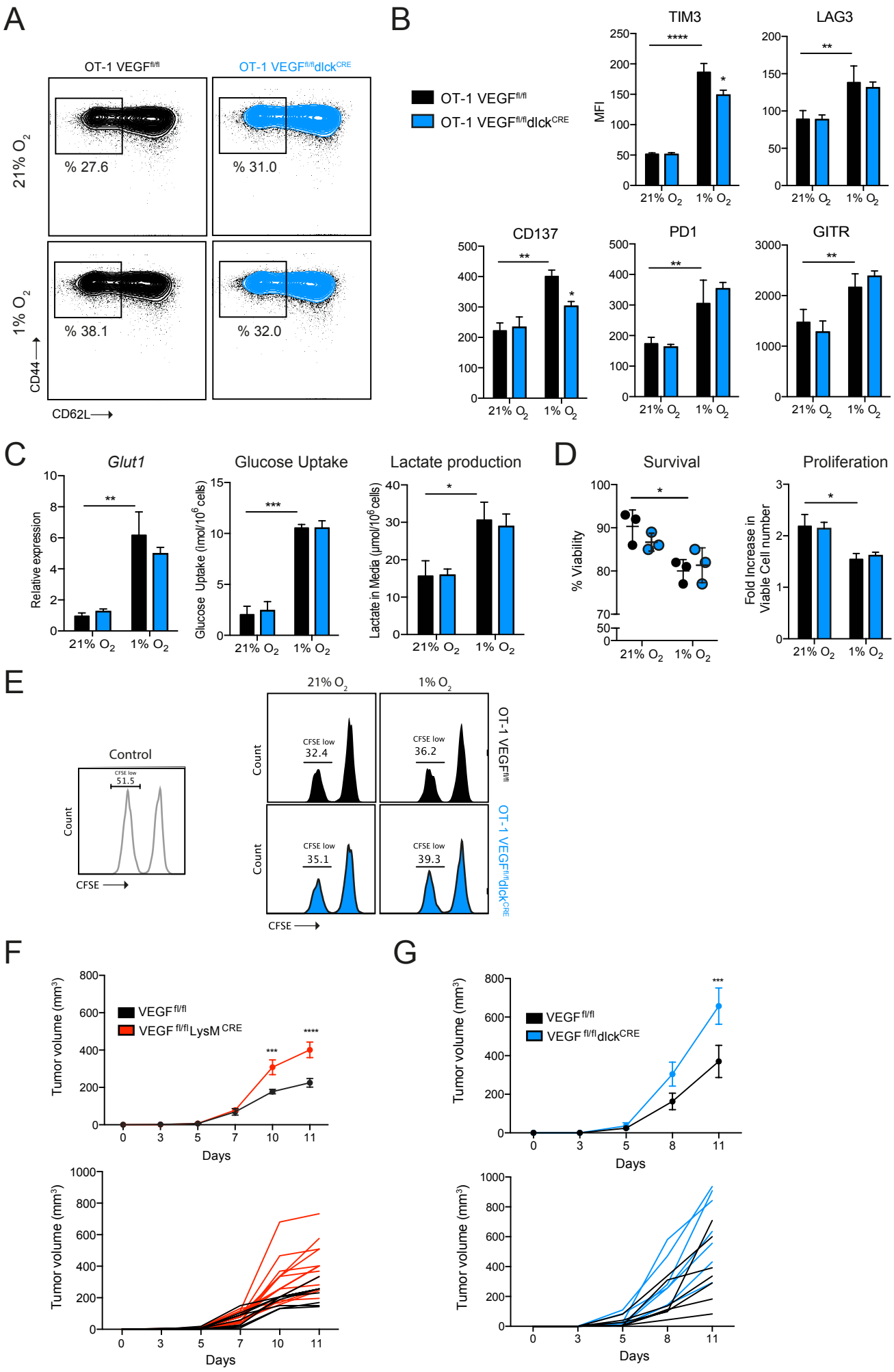
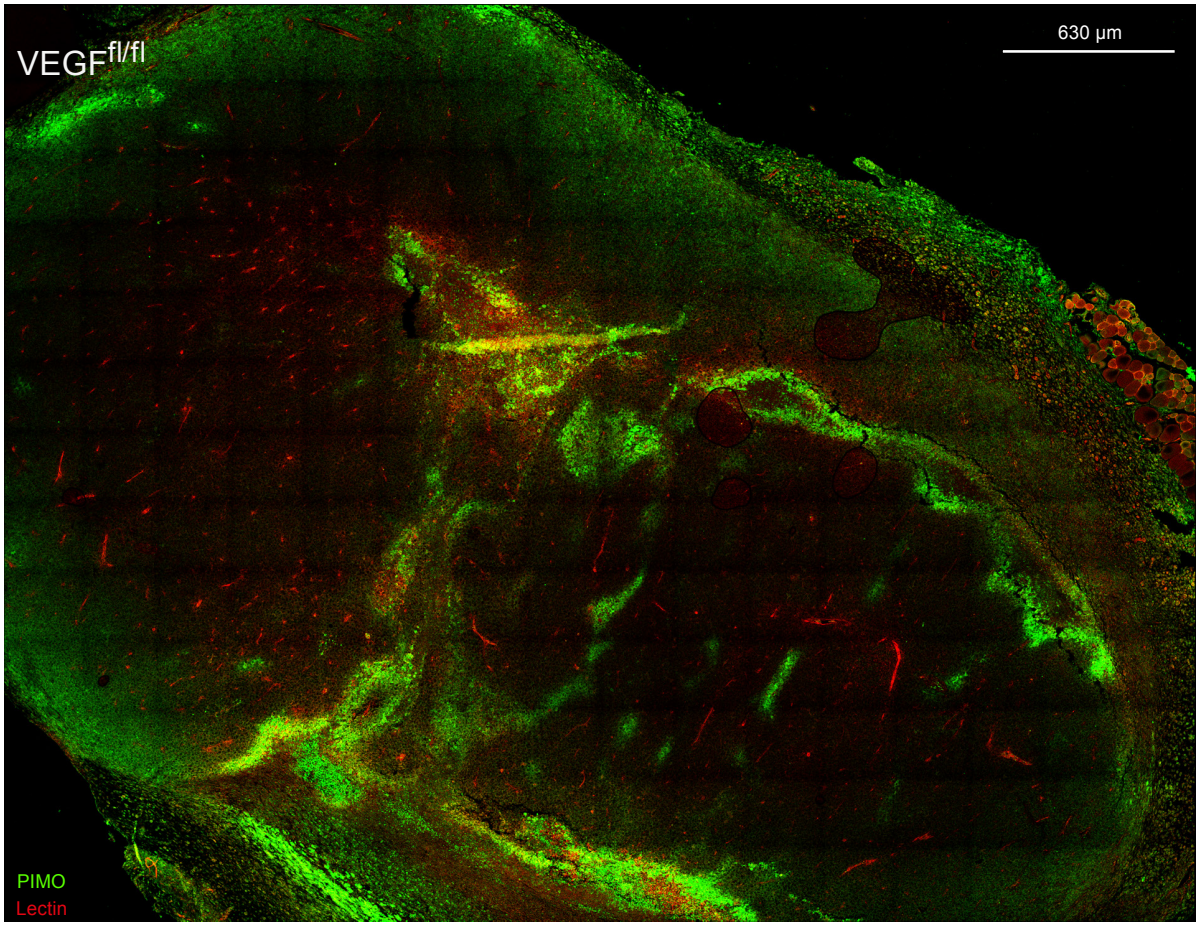

A
dlck CRE
dlck promoter
icre
x
loxP
loxP
VEGF fl/fl
exon2
exon3
exon4
VEGF fl/fl dlck cre
exon2
exon4
B
% Vegfa deletion efficiency
VEGFfl/fldlckCRE
C
Vegfa relative expression
21% O2
1% O2
VEGFfl/fl
VEGFfl/fldlckCRE
D
Amount of VEGF-A in Media (pg/mL)
21% O2
1% O2
E
CD69
CD25
CD62L
MFI
21% O2
1% O2
F
VEGFfl/fl
VEGFfl/fldlckCRE
CD8+ 2.98
CD4+CD8+ 77.9
CD4+ 9.92
CD8+ 3.26
CD4+CD8+ 77.4
CD4+ 9.69
CD8
CD4
% of total thymocytes
CD4+CD8-
CD4+CD8+
CD4-CD8+
G
VEGFfl/fl
VEGFfl/fldlckCRE
CD8+ 13.9
CD4+ 19.1
CD8+ 15.9
CD4+ 18.8
CD8
CD4
% of total splenocytes
CD4+
CD8+
H
VEGFfl/fl
VEGFfl/fldlckCRE
63.3
15.8
15.1
5.79
64.5
18.2
11.6
5.63
CD62L
CD44
% of CD8+ T cells in spleen
CD62L+CD44-
CD62L-CD44-
CD62L+CD44+

Figure S5, related to Figure 6.

(A) Schematic diagram and gene deletion strategy to generate $VEGF^{fl/fl}dlck^{CRE}$ mice. (B) Percentage of *Vegfa* deletion efficiency in gDNA from $CD8^+$ CTLs generated *in vitro* after isolation from from $VEGF^{fl/fl}dlck^{CRE}$ mice (n=4, error bars represent SD). (C) Relative expression of *Vegfa* by QRT-PCR on CTLs from mutant and control mice subjected to 24 hr of culture under 21% O_2 or 1% O_2 (n=4, error bars represent SD). (D) Amount of VEGF-A in media of $CD8^+$ CTLs cultured as in (C), (n=4, error bars represent SD). (E) Mean fluorescence intensity for the surface expression of CD69, CD25 and CD62L measured by flow cytometry on $CD8^+$ T cells 48 hr after activation with αCD3/αCD28. (n=4, error bars represent SD). (F) Thymic composition of mutant mice ($VEGF^{fl/fl}dlck^{CRE}$) and control littermates ($VEGF^{fl/fl}$)(n=4, error bars represent SD). (G) Splenic composition of mutant mice ($VEGF^{fl/fl}dlck^{CRE}$) and control littermates ($VEGF^{fl/fl}$) (n=4, error bars represent SD). (H) $CD8^+$ T cell composition in spleen of mutant mice ($VEGF^{fl/fl}dlck^{CRE}$) and control littermates ($VEGF^{fl/fl}$) according to CD62L and CD44 expression(n=4, error bars represent SD).


A
OT-1 VEGF fl/fl
OT-1 VEGF fl/fl dlck CRE
21% O2
1% O2
% 27.6
% 31.0
% 38.1
% 32.0
CD44
CD62L
B
OT-1 VEGF fl/fl
OT-1 VEGF fl/fl dlck CRE
TIM3
LAG3
CD137
PD1
GITR
MFI
21% O2
1% O2
C
Glut1
Glucose Uptake
Lactate production
Relative expression
Glucose Uptake (Imol/10^6 cells)
Lactate in Media (µmol/10^6 cells)
D
Survival
Proliferation
% Viability
Fold Increase in Viable Cell number
E
Control
CFSE low
51.5
Count
CFSE
21% O2
1% O2
CFSE low 32.4
CFSE low 36.2
CFSE low 35.1
CFSE low 39.3
OT-1 VEGF fl/fl
OT-1 VEGF fl/fl dlck CRE
F
VEGF fl/fl
VEGF fl/fl LysM CRE
Tumor volume (mm3)
Days
G
VEGF fl/fl
VEGF fl/fl dlck CRE
Tumor volume (mm3)
Days

H



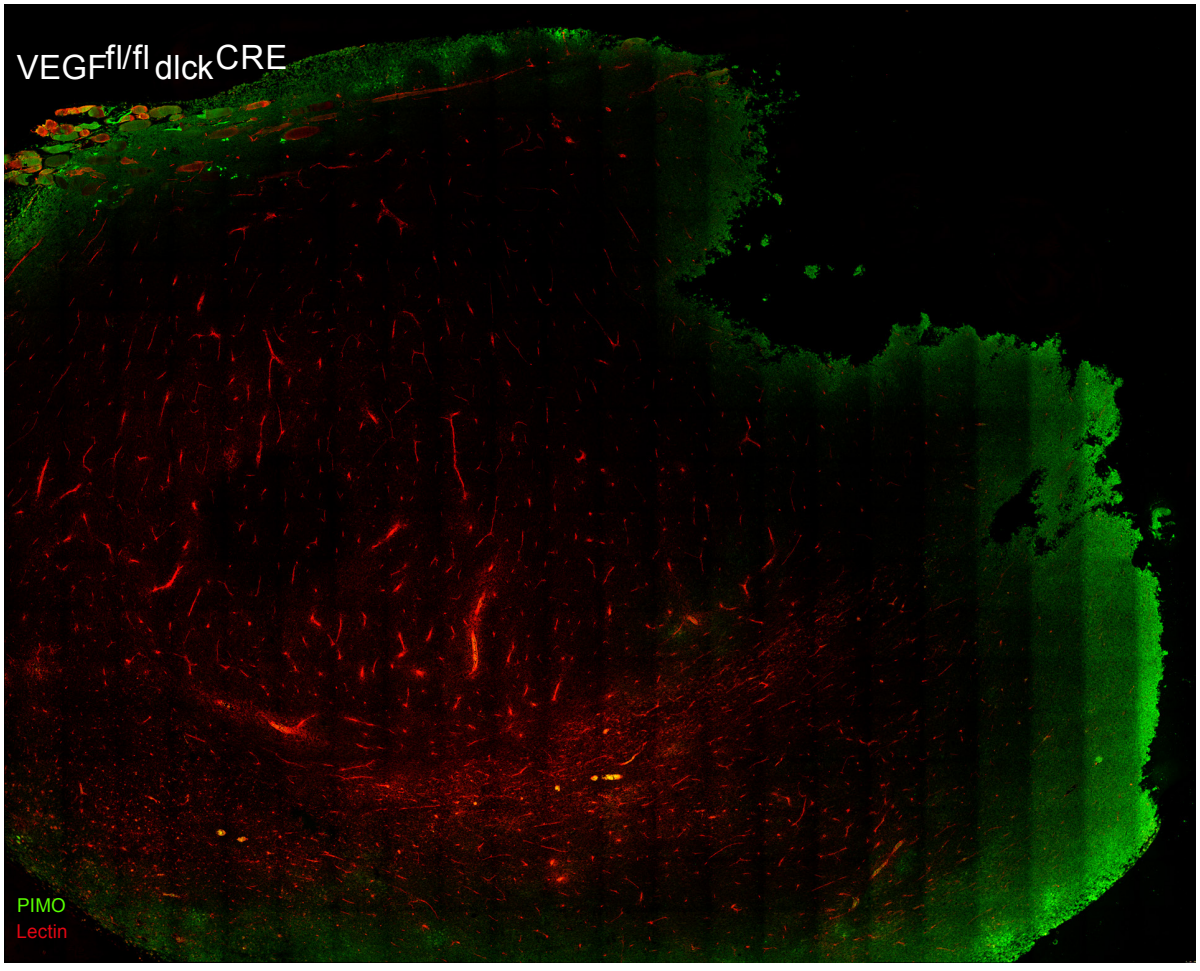


Figure S6, related to Figure 6

(A-D) Splenic $CD8^+$ T cells were isolated from mutant mice (OT-1 $VEGF^{fl/fl}dlck^{CRE}$) and control littermates (OT-1 $VEGF^{fl/fl}$), activated *in vitro* with cognate SIINFEKL peptide and expanded in the presence of IL-2 under 21% O_2 for 5 days and subjected to 21% O_2 or 1% O_2 for the last 24 hours: surface CD62L and CD44 expression by flow cytometry (A), surface expression of costimulatory/checkpoint receptors TIM3, LAG3, CD137, PD-1 and GITR (B), relative mRNA expression of Glut-1 on CTLs (C, Left), glucose uptake and lactate production by CTLs with the indicated genotypes (C, Right) and percent survival (D, Left) and proliferation (D, Right). (n=4, error bars represent SD). (E) *In vitro* killing assay: target cells were pulsed with peptide and stained with $CFSE^{lo}$ and mixed in equal proportions with unpulsed $CFSE^{hi}$ cells. CTLs from mutant or control mice were added to the culture for 4 hours and % of $CFSE^+$ cells were quantified by flow cytometry. (F-G) Representative growth curves of subcutaneously injected LLC tumors in $VEGF^{fl/fl}LysM^{CRE}$ (red, n=13) (F) and $VEGF^{fl/fl}dlck^{CRE}$ (blue, n=7) (G). $VEGF^{fl/fl}$ littermate controls are shown in black, ($0.5x10^6$ tumor cells/mouse). Error bars represent SEM, statistical analysis was performed by two-way ANOVA with Sidak correction for multiple comparisons. (H) Representative immunofluorescence images from LLC tumors for the indicated genotypes (Green= pimonidazole, Red= Tomato lectin-Dylight-594).

Table S2, related to STAR Methods. Primers for qRT-PCR.

Gene	Forward primer	Reverse primer
Rn18s	5'-CGGCGACGACCCATTCGAAC-3'	5'-GAATCGAACCCTGATTCCCCGTC-3'
Hprt	5'- TCAGTCAACGGGGGACATAAA-3'	5'- GGGGCTGTACTGCTTAACCAG-3'
Pgk1	5'-ATTCTGCTTGGACAATGGAGC-3'	5'-AGGCATGGGAACACCATCA-3'
Hk2	5′-TGATCGCCTGCTTATTCACGG-3′	5'-AACCGCCTAGAAATCTCCAGA-3'
Pdk1	5'-GAAGCAGTTCCTGGACTTCG-3'	5'-CCAACTTTGCACCAGCTGTA-3'
Vegfa	5'-CCACGTCAGAGAGCAACATCA-3'	5'-TCATTCTCTCTATGTGCTGGCTTT-3'
Mct4	5'-TCACGGGTTTCTCCTACGC-3'	5'-GCCAAAGCGGTTCACACAC-3'
Hif1a	5'-GAAACGACCACTGCTAAGGCA-3'	5'-GGCAGACAGCTTAAGGCTCCT-3'
Epas1	5'-CAACCTGCAGCCTCAGTGT-3'	5'-CACCACGTCGTTCTTCTCGA-3'

Table S3, related to STAR Methods. Primers for determining deletion efficiency.

Actb Forward	5'-AGAGGGAAATCGTGCGTGA-3'
Actb Reverse	5'-CAATAGTGATGACCTGGCCGT-3'
Actb Probe	5'-[6-FAM] CACTGCCGCATCCTCTTCCTC [BHQ1a-Q]-3'
Vegfa Forward	5'-TGACCATCTGCTTTCGTGACC-3'
Vegfa Reverse	5'-ACTTGTTGCAGGCAGCGG-3'
Vegfa Probe	5'-[6-FAM] TGCTCCCTGGGCTCGACAGGG [BHQ1a-Q]-3'
Hif1a Forward	5' GGTGCTGGTGTCCAAAATGTAG 3'
Hif1a Reverse	5' ATGGGTCTAGAGAGATAGCTCCACA 3'
Hif1a Probe	5' [6-FAM] CCTGTTGGTTGCGCAGCAAGCATT [BHQ1a-Q] 3'
Hif2a Forward	5' TCTATGAGTTGGCTCATGAGTTG 3'
Hif2a Reverse	5' GTCCGAAGGAAGCTGATGG 3'
Hif2a Probe	5' [6-FAM] CCACCTGGA/ZEN/CAAAGCCTCCATCAT [3IABkFQ] 3'