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Supplemental Material to:

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Longitudinal confocal microscopy imaging of tumor eradication following adoptive T-cell transfer

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Legend to Supplemental Figures

Supplementary Figure 1. **Possible scenarios and outcomes of T cell-mediated tumor and/or stromal targeting in the 2C TCR-transgenic SIY/L^d tumor model.** (1) The tumor is eradicated when T cells target both, cancer cells and stromal cells. (2) If T cells target only cancer cells, antigen-loss variants escape and kill the host. (3) T cells only target stromal cells. Cancer cells express antigen but cannot present it on their surface; stromal cells cross-present antigen released by the cancer cells. Tumor growth is arrested and remains in equilibrium for several months. When T cells are depleted, the tumor grows and kills the host.

Supplementary Figure 2. **Tumor growth and T cell-mediated eradication of large, established tumors developing behind the dorsal window. A.** A dorsal window was implanted onto the back skin fold of a C57Bl/6 *Rag1*$^{-/-}$ mouse as described in Methods. Briefly, the front skin layer was removed in a circular area of approximately 1 cm in diameter, leaving the rear skin layer with dermis and fascia intact. Cancer cells were injected at 4 distinct sites in between of the fascia and dermis of rear skin fold in a total volume of 80-100µl PBS. The window was closed with a round glass pane. Within 2-3 weeks, large established tumors developed behind the window. After adoptive transfer of activated, antigen-specific 2C T cells the established tumor regressed and was eliminated within about 10 days, with similar kinetics compared to window-less tumors growing subcutaneously. The upper right pictures (days 21 and 32) correspond to Figure 1F, showing the same mouse. **B.** Growth curves of MC57 SIY-tumors (red) and MC57 control tumor (black) implanted behind windows as described in (a). At day 18 post window/tumor implantation activated 2C T cells were injected into window/tumor bearing mice and tumor volumes measured at indicated times.

Supplementary Figure 3. **Visualization of the tumor stroma. A.** Established, 21 days-old MC57-SIY-Cerulean tumor with dense red stroma and blood vessels; vasculature and blood flow is also

shown by backscatter, bright field, and DiD-labeled red blood cells that were injected intravenously into the tumor-bearing mouse before imaging. Scale bar = 50μm. **B.** Window chamber implantation without cancer cell implantation (only 80μl PBS) were injected in between of the fascia and dermis of rear skin fold [at 4 different sites, each 20μl]) onto the back of a DsRed *Rag1*$^{-/-}$ mouse. Window chamber implantation causes injury and inflammation in response to the surgery which disappears gradually over time. One day post window implantation, injection sites were imaged (left). High numbers of round red host cells were visible. At day 8 post window implantation, the cellular infiltrates have largely disappeared and no cohesive stroma has formed (right). **C.** Longitudinal visualization of tumor stroma formation. Pro4L-SIY-Cerulean cancer cells were injected into a DsRed *Rag1*$^{-/-}$ mouse. Images were taken 14h, 3d, and 7d post window and cancer cell implantation. While 14h post window/cancer implantation, the site is densely infiltrated with round, rapidly moving stromal cells (probably inflammatory cells), 4-8 days later the number of these cells is greatly reduced and many stromal cells acquire a fibroblastoid shape.

Supplementary Figure 4. **Quantitative analysis of T-cell motility in an antigen-sensitized or non-sensitized tumor microenvironment. A.** Arrest coefficient, diffusion coefficient, and average velocity of 2C T cells in antigen-positive Pro4LSIY-Cerulean (n=2; blue and green) and antigen-negative Pro4L-Cerulean tumors (n=1; black) determined by longitudinal imaging over several days. Red lines indicate mean value, bars indicate SD. **B.** Data presented in (a) showing individual 2C T cells. Each dot represents an individual 2C T cell. Bars indicate SD, red lines indicate mean. The data represent a total of 3 mice (2 Ag-positive, 1 Ag-negative) followed over time. A total of 21 movies were analyzed (16 Ag-positive, 5 Ag-negative. 14-130 tracks were analyzed per movie. **C.** Arrest coefficient, diffusion coefficient, and average velocity of 2C T cells in antigen-positive MC57-SIY-Cerulean and antigen-negative MC57-Cerulean tumors. Each dot represents an individual 2C T cell; red lines indicate mean; black bars indicate StDev. Data are from one experiment with one mouse per group analyzed

concurrent and repeatedly. 44 tracks from one movie for the antigen-positive mouse and 36 tracks from 2 movies for the antigen-negative mouse were analyzed. $^{***}p<0.0001$. For (A)-(C) T cell tracks with > 950 sec (15 minutes) were considered. **D.** No evidence of imaging-induced phototoxicity or damaged T cell migration. Comparison of the average velocities of adoptively transferred CD8 T cells at the beginning (during the first 38 minutes; open circles) and at the end (during the last 38 minutes; solid circles) of a 2-hours long imaging procedure. T cells in the imaged tumor area reveal the same average velocity at the beginning and at the end of the movie (over 100 minutes later).

Supplementary Figure 5. **Vessel leakiness and vessel destruction in antigen-positive, solid tumors post transfer of antigen-specific CD8 T cells. A,B.** C57Bl/6 *Rag1*$^{-/-}$ mice bearing (**A**) an established, 20 days-old antigen-positive MC57-SIY-Cerulean tumor, or (**B**) 20 days-old, established antigen-negative MC57-Cerulean tumor, growing behind windows, were injected with *in vitro* activated 2C EYFP T cells. To directly measure blood flow in tumors, DiD-labeled red blood cells were injected 2 days post T cell transfer. Tumors were imaged longitudinally post T cell transfer at indicated time points. No changes in blood flow dynamics were observed in antigen-negative MC57-Cerulean tumors and the vasculature was intact 4 days post T cell transfer as evidenced by vessel-localized DiD-labeled red blood cells and intact vessel structures throughout the tumor. However, vessels in MC57-SIY-Cerulean tumors were leaky and destroyed, evidenced by the cessation of blood flow, disappearance of vessel structures and the diffuse distribution of DiD-labeled red blood cells throughout the entire tumor area. Scale bar = 75 μm. Images are corresponding to Supplementary Videos 10, 11 and 13. For better visualization of DiD-labeled red blood cells, maximal projections are shown.

Supplementary Figure 6. **Longitudinal imaging of a 4-color *in vivo* tumor model reveals bystander elimination of antigen-loss variants through stromal targeting. A.** Scheme of the 4-color tumor model. Model 1: Antigen-positive MC57-SIY-Cerulean cancer cells are mixed with 5% of MC57-

EGFP cancer cells mimicking ALV; Model 2: Antigen-positive MC57-L^d-Cerulean cancer cells are mixed with 5% of MC57-EGFP cancer cells mimicking ALV. Mixtures are transplanted into DsRed *Rag1*$^{-/-}$ mice. **B.** Images of tumors before (left panel) and after adoptive transfer of *in vitro* activated 2C EYFP CD8 T cells and subsequent T cell mediated tumor destruction (panels to the very right represent enlarged overlays of all 4 colors/channels: yellow T cells, blue Ag-positive cells, green ALV, and red stroma). In Model 1 both, green ALV and MC57-SIY-Cerulean cancer cells are eventually eliminated post T cell transfer, in contrast to model 2, where only MC57-L^d-Cerulean are eliminated and ALV in the microenvironment persist, and continue to grow. **C.** Area occupied by tumor (blue) and ALV cells (green) before and after T cell transfer (3 days post T cell transfer for SIY model; 4 days post T cell transfer for the L^d model). Average velocities (**D**), arrest coefficients (**E**), and diffusion coefficients (**F**) of T cells in microenvironments of MC57-SIY or MC57-L^d tumors are graphed. In **D** and **F**, each dot represents an individual T cell. Red lines indicate mean; black bars indicate SD ($^{***}p<0.0001$). **G.** Representative displacement tracks from 2C T cells in MC57-SIY or MC57-L^d tumors. Data are representative of 2 independent experiments with a total of 4 mice (2 SIY-models, 2 L^d-models); a total of 9 movies were evaluated - 4 movies for the SIY-model, 5 for the L^d-model. Only T cell tracks > 950 sec (15 minutes) were considered for analysis. 34-77 tracks were analyzed per movie.

Legends to Supplementary Videos

Supplementary Video 1: Fourteen hours post window implantation and cancer cell transplantation, blue MC57-SIY-Cerulean cancer cells and rapidly moving, infiltrating DsRed-host cells are imaged.

Supplementary Video 2: Day 8 post window implantation and cancer cell transplantation DsRed-host stromal cells have a non-migratory fibroblastoid shape and form a cohesive network together with blue MC57-SIY-Cerulean cancer cells.

Supplementary Video 3: Three days post adoptive transfer of *in vitro* activated 2C EYFP $CD8^+$ T cells, T cells extravasate and infiltrate a MC57 SIY-Cerulean tumor (host: DsRed *Rag1*$^{-/-}$ mouse). Movie corresponds to Figure 3B.

Supplementary Video 4: Six days post T cell transfer, MC57 SIY-Cerulean blue cancer cells are being destroyed as evidenced by membrane blebbing (middle circle). Red stromal cells engulf blue, apoptotic cancer material (left circle). Host: DsRed *Rag1*$^{-/-}$ mouse; 2C EYFP $CD8^+$ T cells (green).

Supplementary Video 5: 2C EYFP $CD8^+$ T cells within the tumor microenvironment of MC57 SIY-Cerulean tumors during the elimination phase, six days post adoptive transfer into tumor-bearing mice. Host: DsRed *Rag1*$^{-/-}$ mouse.

Supplementary Video 6: 8 days post adoptive transfer, 2C EYFP $CD8^+$ T cells in the microenvironment of a MC57 SIY-Cerulean blue tumor; cancer cells are largely eliminated, and tumor-specific 2C T cells remain in the microenvironment and remain engaged with red stromal cells (host: DsRed *Rag1*$^{-/-}$ mouse). See corresponding quantitative analyses presented in Figures 3I,J.

Supplementary Video 7: 3-4 days after cancer cell elimination, 2C $CD8^+$ T cells (green) within the microenvironment (red) of rejected tumors eventually regain their amoeboid form and move rapidly within the microenvironment. See corresponding quantitative analyses presented in Figure 3J.

Supplementary Video 8: 2C EYFP $CD8^+$ T cells in the microenvironment of antigen-positive, 27 days-old Pro4L-SIY-Cerulean tumors. T cell motility is dependent on cognate antigen expressed and released by cancer cells and cross-presented by sensitized stromal cells as T cells have a higher arrest coefficient and lower mean velocities (see representative, quantitative analyses presented in Figures 4B-4D) compared to T cells in antigen-negative Pro4L-Cerulean tumors (Video 9). Video was taken 3 days post T cell transfer (and 27 days post window/tumor implantation).

Supplementary Video 9: 2C EYFP $CD8^+$ T cells in antigen-negative established, 17 days-old Pro4L-Cerulean tumors. T cells have a low arrest coefficient and high mean velocities (see representative, quantitative analyses presented in Figures 4B-D). Host: DsRed *Rag1*$^{-/-}$ mouse. Video was taken 5 days post T cell transfer (and 17 days post window/tumor implantation).

Supplementary Video 10: A C57Bl76 *Rag1*$^{-/-}$ host mouse bearing an established, 20 days-old antigen-positive MC57-SIY-Cerulean tumor (blue) growing behind a window was injected with *in vitro* activated 2C EYFP T cells (green). DiD-labeled red blood cells (red) were injected 2 days post T cell transfer and tumor was imaged shortly after. Vasculature/blood flow is intact as evidenced by vessel-localized DiD-labeled red blood cells flowing through the tumor vessels. Video is corresponding to Supplementary Figure 5A, top panel.

Supplementary Video 11: A C57Bl76 *Rag1*$^{-/-}$ host mouse bearing an established, 20 days-old antigen-positive MC57-SIY-Cerulean tumor (blue) growing behind a window was injected with *in vitro* activated 2C EYFP T cells (green). DiD-labeled red blood cells (red) were injected 2 days post T cell transfer and tumor was imaged 3 days after. Vessels are leaky and destroyed, demonstrated by the ablation of blood flow, disappearance of intact vessel structures and the diffuse distribution of DiD-labeled red blood cells throughout the entire tumor area. T cells are captured in this microenvironment. Video is corresponding to Supplementary Figure 5A, bottom panel.

Supplementary Video 12: A DsRed *Rag1*$^{-/-}$ mouse bearing an established, antigen-positive MC57 SIY-Cerulean tumor behind a window was injected with *in vitro* activated 2C EYFP CD8 T cells at day 18 of tumor growth. DiD-labeled red blood cells were injected at the day of T cell transfer. Tumor was imaged longitudinally and z-stacks were acquired. Shown are 3D reconstruction images (rotational views) of the volume occupied by DiD-red blood cells at the indicated time points after T cell transfer [day 1.5 (left); day 2.0 (middle); day 2.5 (right)]. The 3D reconstruction images of the vessel

morphology illustrate the loss of defined vascular structures over time after T cell transfer. Data are representative of two independent experiments with multiple regions followed in each experiment (video corresponds to Figure 5B). A scale bar and time points are shown at the beginning of the movie.

Supplementary Video 13: A B6 *Rag1*$^{-/-}$ host mouse bearing an established, 20 days-old antigen-negative MC57-Cerulean tumor (blue) growing behind a window was injected with *in vitro* activated 2C EYFP T cells (green). DiD-labeled red blood cells (red) were injected 2 days post T cell transfer and tumor was imaged 4 days after. T cells are moving rapidly and tumor vasculature is intact 4 days post T cell transfer as evidenced by vessel-localized DiD-labeled red blood cells, blood flow and intact vessel architecture throughout the tumor. Video corresponds to Supplementary Figure 5B, bottom panel.

Supplementary Video 14: 4-5 days post T cell transfer, 2C EYFP CD8^{+} T cells destroy MC57-SIY-Cerulean cancer cells. A few MC57-DsRed cancer cells, mimicking antigen-loss variants (that were initially mixed in a 1:20 ratio, and implanted together with antigen-positive MC57-SIY-Cerulean cancer cells) are still visible, but will eventually die (see corresponding Figure 6D, image 4). 2C T cells remain in the sensitized, targetable stroma and have a low average velocity and a high arrest coefficient (host: *Rag1*$^{-/-}$ mouse). Video corresponds to Figure 6 and quantitative analyses are presented in Figures 6F,G.

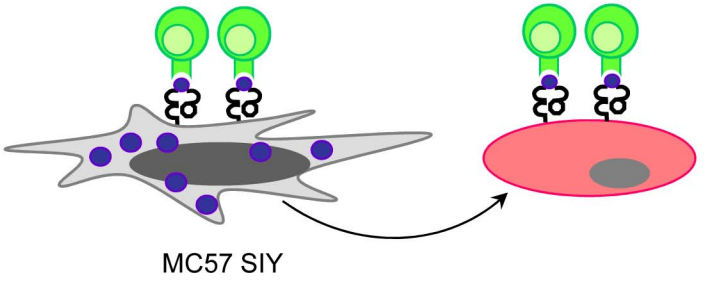
Supplementary Video 15: 4-5 days post adoptive T cell transfer, 2C EYFP CD8^{+} T cells destroy antigen-positive MC57-L^{d}-Cerulean cancer cells. MC57-DsRed cancer cells, mimicking antigen-loss variants (that were initially mixed in a 1:20 ratio, and implanted together with antigen-positive MC57-L^{d}-Cerulean cancer cells) are remaining and eventually will continue to grow (see corresponding Figure 6D, panel 4). 2C T cells remain in the non-sensitized stroma and have a higher average velocity

and a lower arrest coefficient compared to the T cells in Video 14. Video corresponds to Figure 6 and quantitative analyses presented in Figures 6F and 6G. (Host: C57Bl/6 *Rag1*$^{-/-}$ mouse).

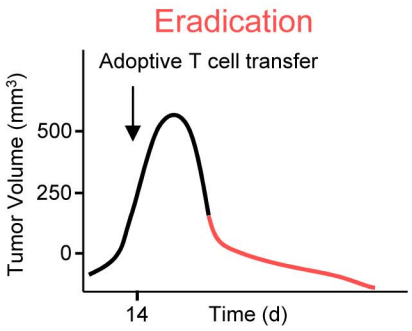
Supplementary Video 16: Antigen-positive MC57-SIY-Cerulean cancer cells (blue) were mixed with 5% of MC57-EGFP (green) cancer cells (mimicking ALV) and transplanted into a DsRed *Rag1*$^{-/-}$ mouse (red). Once tumor was established, *in vitro* activated 2C EYFP CD8^{+} T cells (yellow) were adoptively transferred. T cells eliminated blue MC57-SIY-Cerulean and are arrested in the microenvironment, engaging with sensitized stromal cells cross-presenting tumor antigen. Video was taken 4 days post T cell transfer. Video corresponds to Supplementary Figure 6 and quantitative analyses of T cell motility are presented in Supplementary Figures 6C-6G. EGFP to EYFP bleed correction was performed but was not complete for the fastest moving EYFP-cells (visible as green "halos" around some EYFP-yellow cells).

Supplementary Video 17: Antigen-positive MC57-L^{d}-Cerulean cancer cells (blue) were mixed with 5% of MC57-EGFP (green) cancer cells (mimicking ALV), and transplanted into a DsRed *Rag1*$^{-/-}$ mouse (red). Once tumor was established, *in vitro* activated 2C EYFP CD8^{+} T cells (yellow) were adoptively transferred. T cells eliminated mostly blue MC57-L^{d}-Cerulean cancer cells and MC57-EGFP cancer cells remain and continue to grow (see Supplementary Figure 6C). Video was taken 8 days post T cell transfer and corresponds to Supplementary Figure 6. T cells are not arrested and do not engage with red stromal cells, evidenced by low arrest coefficient and high mean velocities. Quantitative analyses of T cell motility are presented in Supplementary Figures 6D-G.

1 Cancer and stromal cells are T cell targets

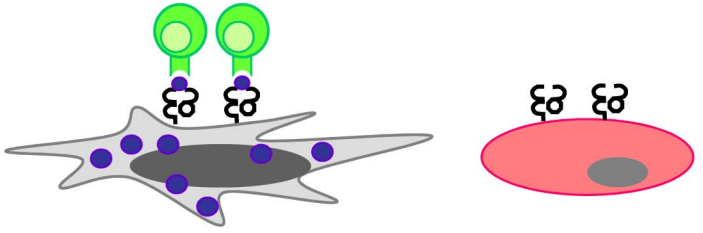


MC57 SIY

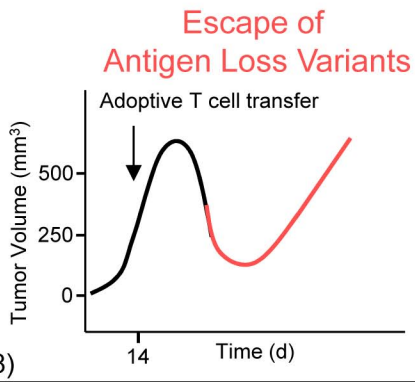


Spiotto MT *et al*, Immunity (2002);Zhang B *et al*, J Exp Med (2007); Engels B *et al*, Cancer Cell (2013)

2 Only cancer cell is T cell target

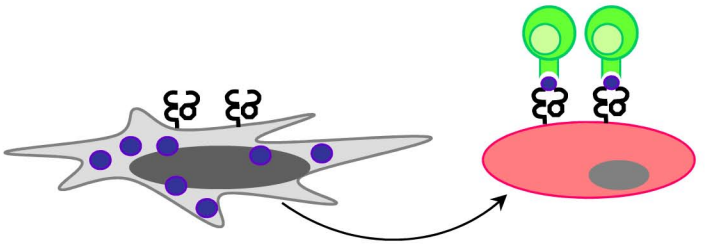


MC57 L^d

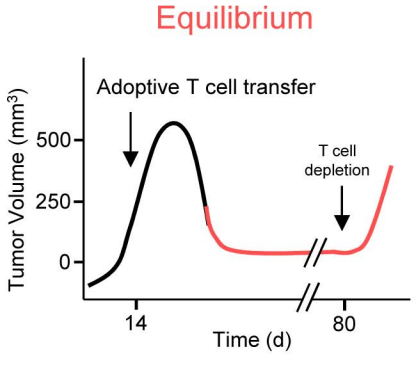


Spiotto MT *et al*, Nat. Med. (2004); Zhang B *et al*, J Clin Invest (2008)

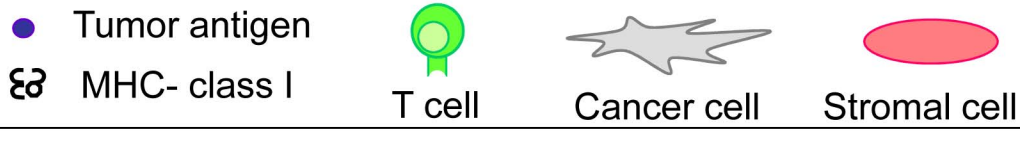
3 Only stromal cell is T cell target



Pro4L SIY



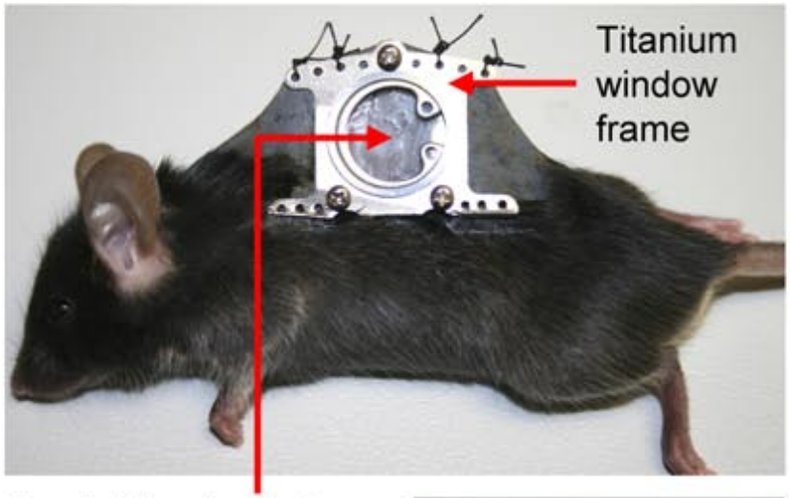
Zhang B *et al*, Cancer Res (2008)



a

Day 0

Window chamber implantation

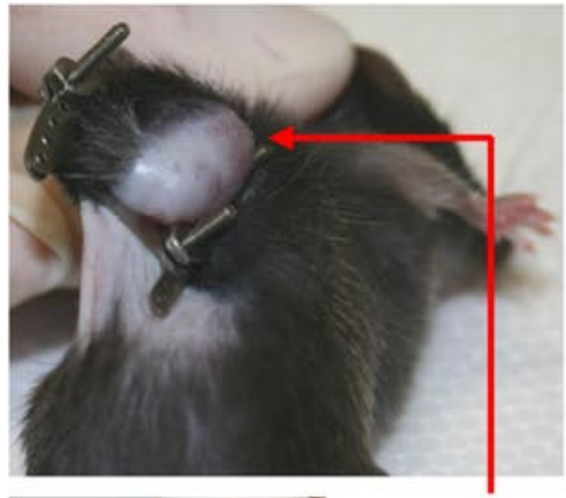


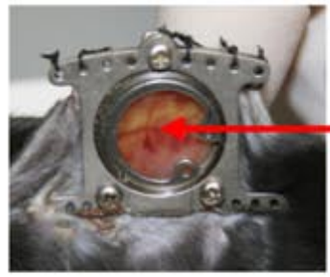
Front side of window: cancer cells are injected between fascia and dermis of rear skin fold; fascia is then covered with microscope glass and C-ring is installed for stabilization.

Backside of implanted skin fold window.

Day 21

Established tumor



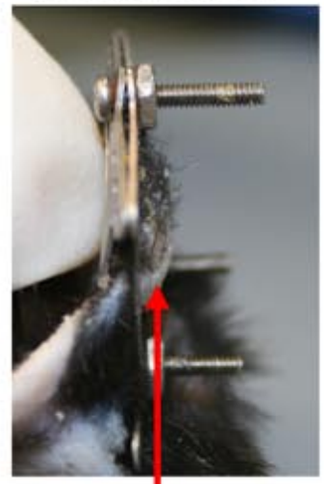


Backside: established tumor

Tumor tissue with vasculature visible through glass window

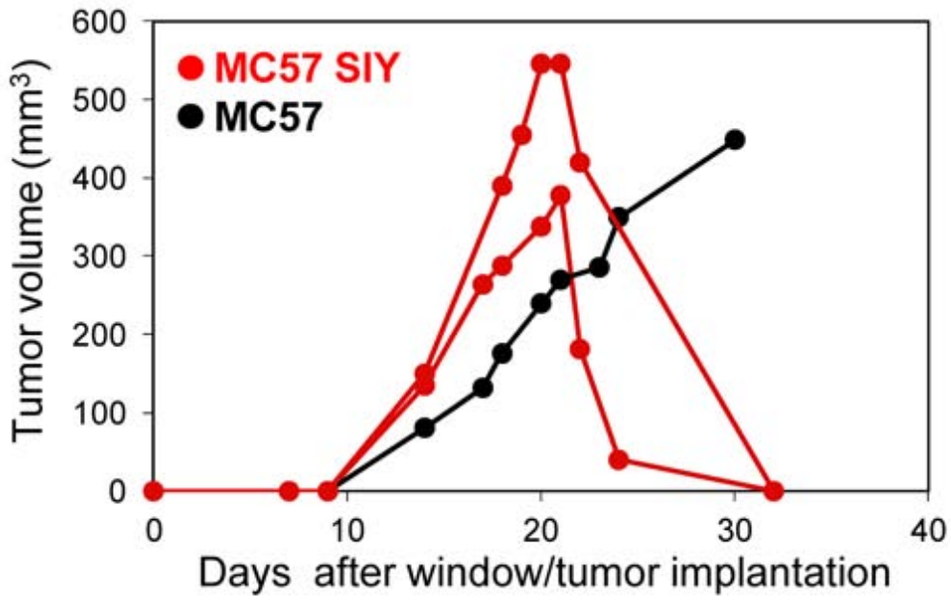
Day 32

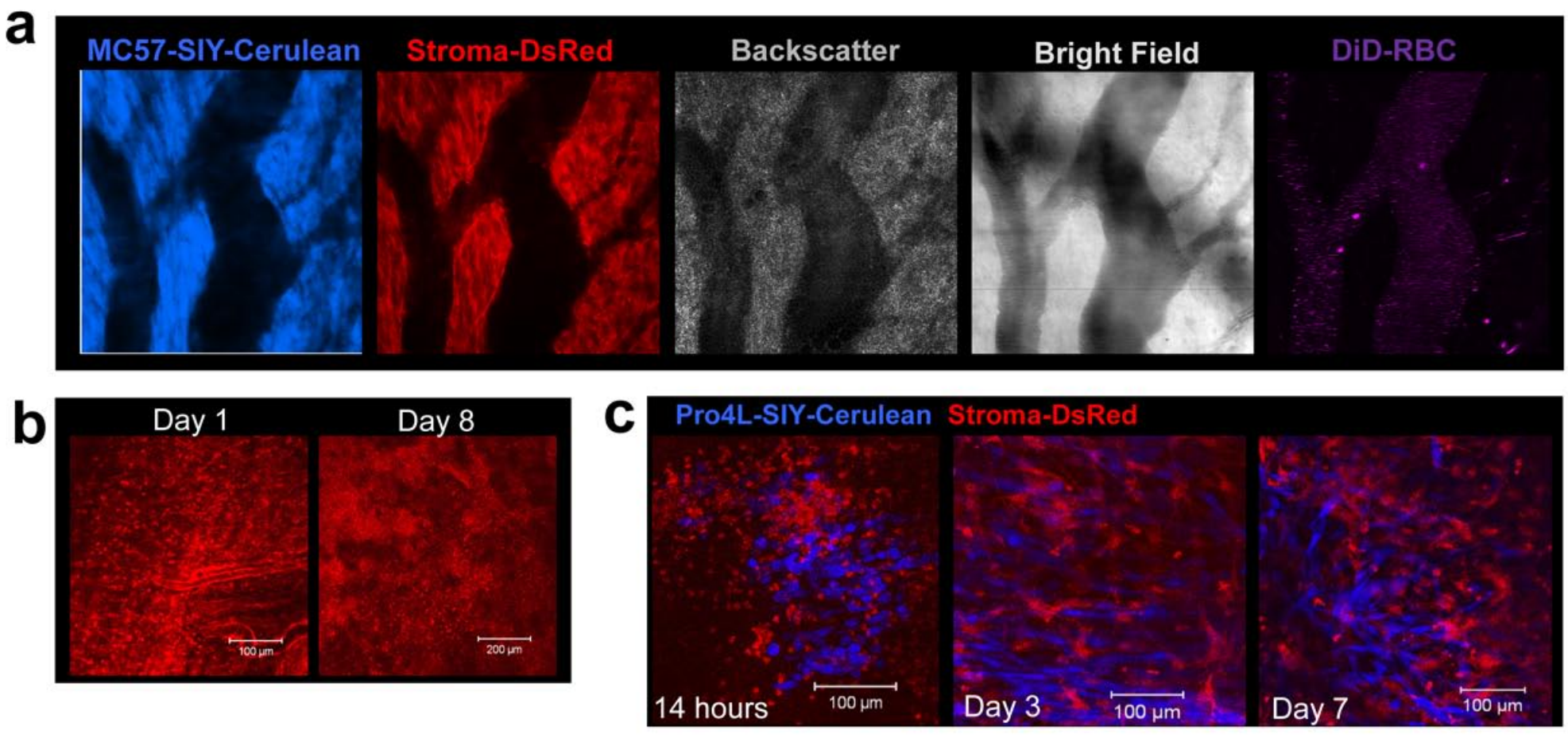
Tumor eradication after adoptive T cell transfer

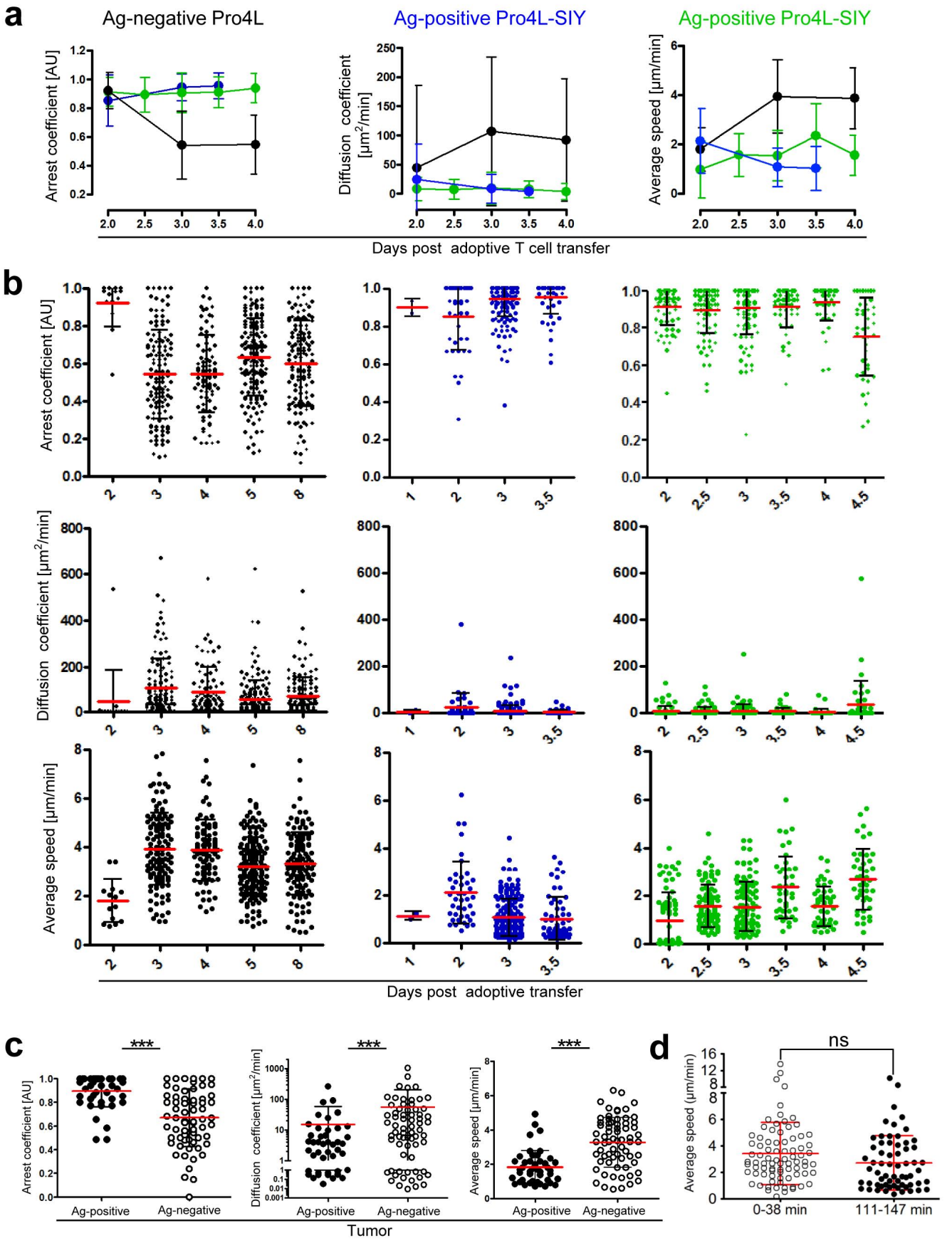


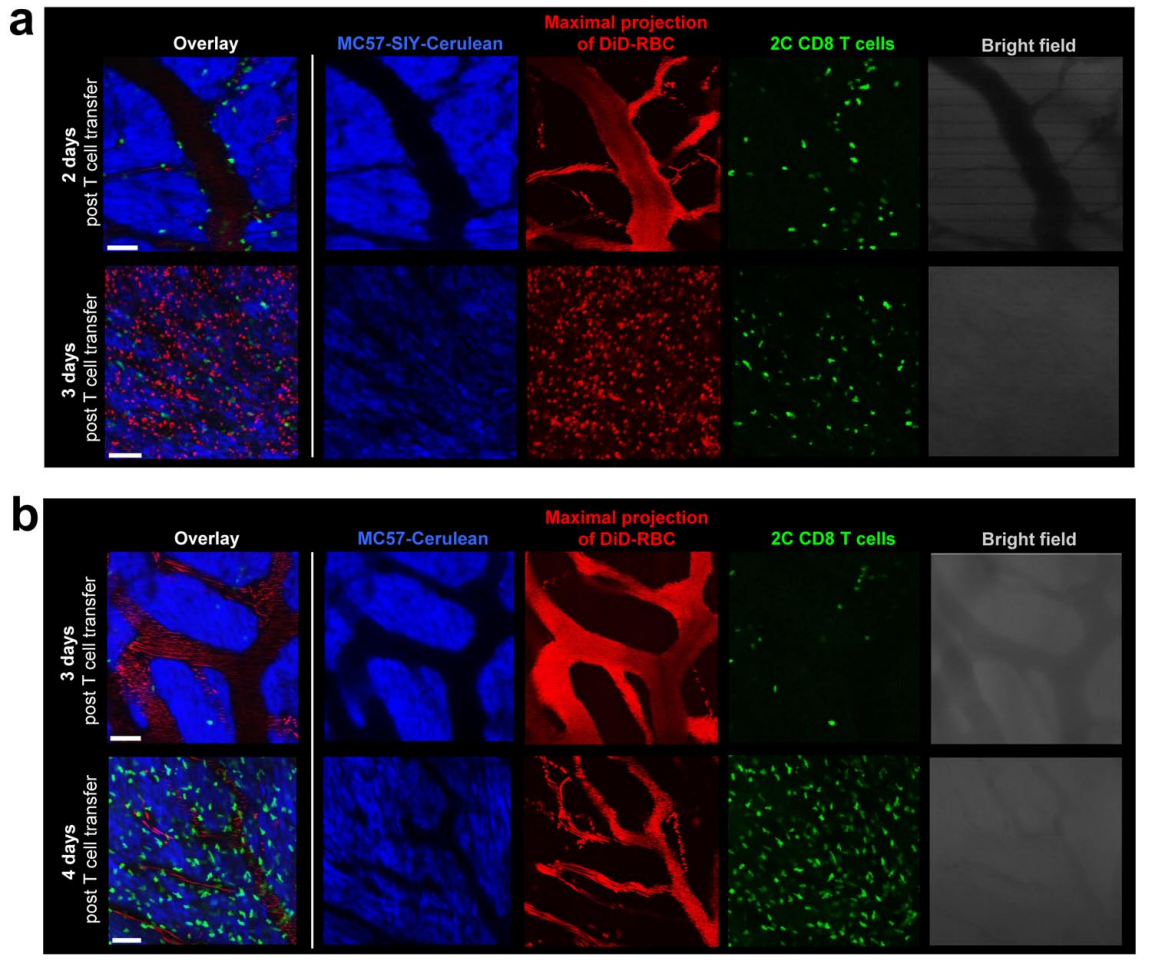
Tumor regresses after adoptive T cell transfer

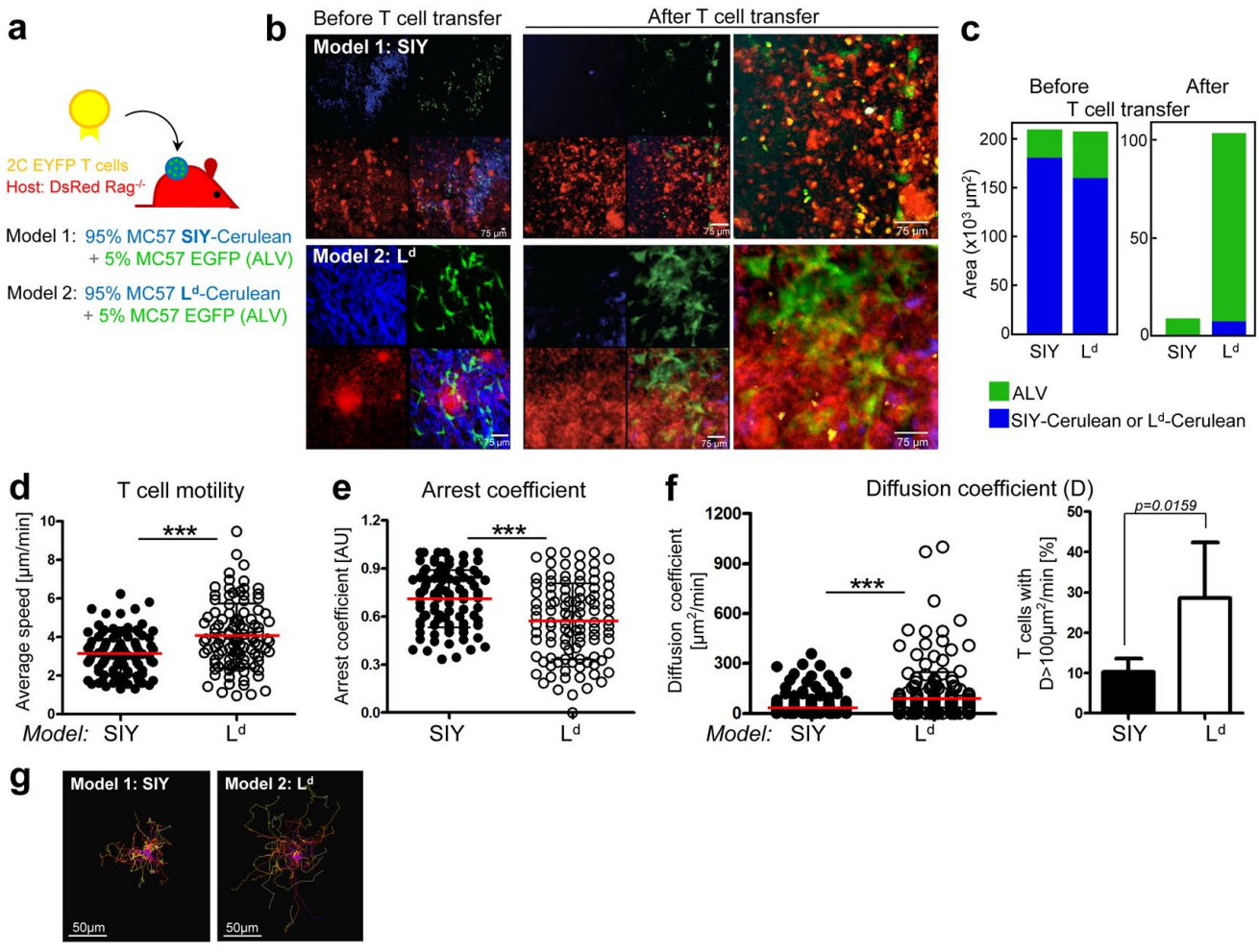
b




a
MC57-SIY-Cerulean
Stroma-DsRed
Backscatter
Bright Field
DiD-RBC
b
Day 1
Day 8
100 µm
200 µm
c
Pro4L-SIY-Cerulean
Stroma-DsRed
14 hours
100 µm
Day 3
100 µm
Day 7
100 µm


a
Ag-negative Pro4L
Ag-positive Pro4L-SIY
Ag-positive Pro4L-SIY
Arrest coefficient [AU]
Diffusion coefficient [µm²/min]
Average speed [µm/min]
Days post adoptive T cell transfer
b
Arrest coefficient [AU]
Diffusion coefficient [µm²/min]
Average speed [µm/min]
Days post adoptive transfer
c
***
***
***
Arrest coefficient [AU]
Diffusion coefficient [µm²/min]
Average speed [µm/min]
Ag-positive
Ag-negative
Tumor
d
ns
Average speed (µm/min)
0-38 min
111-147 min


a
Overlay
MC57-SIY-Cerulean
Maximal projection of DiD-RBC
2C CD8 T cells
Bright field
2 days post T cell transfer
3 days post T cell transfer
b
Overlay
MC57-Cerulean
Maximal projection of DiD-RBC
2C CD8 T cells
Bright field
3 days post T cell transfer
4 days post T cell transfer


a
2C EYFP T cells
Host: DsRed Rag-/-
Model 1: 95% MC57 SIY-Cerulean
+ 5% MC57 EGFP (ALV)
Model 2: 95% MC57 Ld-Cerulean
+ 5% MC57 EGFP (ALV)
b
Before T cell transfer
After T cell transfer
Model 1: SIY
Model 2: Ld
75 µm
c
Before
After
T cell transfer
Area (x10³ µm²)
200
150
100
50
0
100
50
0
SIY
Ld
SIY
Ld
ALV
SIY-Cerulean or Ld-Cerulean
d
T cell motility
Average speed [µm/min]
10
8
6
4
2
0
***
Model: SIY Ld
e
Arrest coefficient
Arrest coefficient [AU]
1.2
0.9
0.6
0.3
0.0
***
SIY
Ld
f
Diffusion coefficient (D)
Diffusion coefficient [µm²/min]
1200
900
600
300
0
***
Model: SIY Ld
T cells with D>100µm²/min [%]
50
40
30
20
10
0
p=0.0159
SIY
Ld
g
Model 1: SIY
Model 2: Ld
50µm
50µm