Figures and figure supplements

Long-term intravital imaging of the multicolor-coded tumor microenvironment during combination immunotherapy

Shuhong Qi et al
Figure 1. ‘Immunosuppressive ring’ formed by Tregs in CFP-B16 tumor-bearing mice, which inhibited the anti-tumor efficacy of the adoptive CTLs. (A) Tumor growth curves for CFP-B16 tumors of mice treated with ACT or PBS control. The data are represented as the mean ± SEM tumor volume (n = 12–14, three independent experiments). ns: not significant, (Figure 1—source data 1). (B) Density of carboxyfluorescein succinimidyl ester (CFSE)-labeled CTLs within different organs (tumor-draining lymph nodes (TDLNs), non-tumor-draining lymph nodes (NDLNs), spleens, and tumors). The density was determined by counting the number of CFSE-labeled CTLs per mm² on frozen tissue sections. The data are represented as the mean ± SEM (n = 18–22 fields, 0.18 mm² per field) from three independent experiments. *p<0.05, **p<0.01, ***p<0.001, (Figure 1—source data 2). (C) Large-field intravital images of an ‘immunosuppressive ring’ around the CFP-B16 tumor. Blue – CFP-B16 tumor; red – Tregs (Foxp3-mRFP cells); green – Figure 1 continued on next page.
Figure 1 continued

CFSE-labeled CTLs. The left panel shows different single color channels of the tumor microenvironment, and the right panel shows the three color channels merged. Scale bar: 500 µm. (D) Schematic diagram of the ‘immunosuppressive ring’ in the tumor microenvironment.
DOI: 10.7554/eLife.14756.003

The following source data is available for figure 1:

Source data 1. Tumor growth curves for CFP-B16 tumors of mice treated with ACT or PBS.
DOI: 10.7554/eLife.14756.004

Source data 2. Density of CFSE-labeled CTLs within different organs.
DOI: 10.7554/eLife.14756.005
Figure 1—figure supplement 1. Establishment and characterization of CFP-B16 tumor-specific CTLs. (A) CFSE- and propidium iodine (PI)-based assays to assess the cytotoxicity of CTLs added to the CFP-B16 tumor cells or added to the splenocytes by flow cytometry, and the control group which was composed of splenocytes added to the CFP-B16 tumor cells. The data are represented as the mean ± SD from three independent experiments. (B) Analysis and characterization of the in vitro-activated CTLs. The data are representative of similar results from three independent experiments. DOI: 10.7554/eLife.14756.006
Figure 1—figure supplement 2. Fluorescence microscopy images showing the distribution of CFSE-labeled CTLs in different organs. Representative sections of tumor-draining lymph node (TDLN), non-tumor-draining lymph node (NDLN), spleen and tumor tissues were collected from tumor-bearing mice on Days 1–3 (early stage) and Days 4–6 (late stage) after the adoptive transfer of CTLs. The top row represents the early stage, and the bottom row represents the late stage. CFSE-labeled adoptively transferred CTLs are shown in green. Scale bars: 50 μm.

DOI: 10.7554/eLife.14756.007
**Figure 1—figure supplement 3.** Long-term and large-field imaging of the process by which Tregs formed an immunosuppressive ring. CFP-B16 tumor is shown in blue (CFP), Tregs are shown in red (mRFP), and adoptively transferred CTLs are shown in green (CFSE, rarely observed). Top row: large-field images; scale bar: 500 μm. Bottom row: images of the region of interest; scale bar: 100 μm.

DOI: 10.7554/eLife.14756.008
Figure 2. Synergistic effect of CTX and the adoptive CTLs (ACT) on CFP-B16 tumor immunotherapy. (A) Growth curves for the CFP-B16 tumors treated with ACT, CTX or CTX-ACT and the PBS control. The data are represented as the mean ± SEM tumor volume (n = 12–14, three independent experiments). ns: not significant, ***p<0.001, (Figure 2—source data 1). (B) Intravital confocal fluorescence imaging of Tregs (red) at the tumor periphery (left panel), and immunohistochemistry (right panel) of the CFP-B16 tumor tissues after ACT (top row) or CTX-ACT (bottom row) treatment. Scale bars: 100 μm. The arrows indicate Tregs. (C) Long-term intravital imaging of the multicolor-coded tumor environment in CTX-ACT-treated mice. Red – Tregs (Foxp3-mRFP); green –CSFE-labeled CTLs; blue –CFP-B16 tumor. Top row: large-field images; scale bar: 500 μm. Bottom row: images from the region of interest in the top row; scale bar: 100 μm. The imaging data are representative of similar results from 3–5 mice in two independent experiments.

DOI: 10.7554/eLife.14756.009

The following source data is available for figure 2:

**Source data 1.** Growth curves for the CFP-B16 tumors treated with ACT, CTX, CTX-ACT or PBS control.

DOI: 10.7554/eLife.14756.010
Figure 2—figure supplement 1. Evaluation of the effect of different doses of CTX and CTX combined with ACT on CFP-B16 tumor growth in vivo. (A, B) Growth curves for the CFP-B16 tumors in (A) C57BL/6 and (B) BALB/c nude mice treated with different doses of CTX (50, 100 or 150 mg kg\(^{-1}\)) and different doses of CTX (50, 100 or 150 mg kg\(^{-1}\)) combined with ACT, and PBS control. The data are represented as the mean ± SEM tumor volume (n = 9–10, two independent experiments). ***p<0.001, ns: not significant, (Figure 2—figure supplement 1—source data 1).

DOI: 10.7554/eLife.14756.011

The following source data is available for figure 2:

Figure supplement 1—Source data 1. Growth curves for the CFP-B16 tumors in C57BL/6 and BALB/c nude mice treated with different doses of CTX, different doses of CTX combined with ACT treatment and PBS control. DOI: 10.7554/eLife.14756.012
Figure 2—figure supplement 2. Quantification of the intravital imaging of Tregs and adoptive CTLs in ACT- and CTX-ACT-treated mice. (A) Density of Tregs and (B) adoptive CTLs were determined by counting the number of mRFP-Tregs and CFSE-labeled CTLs per mm$^2$ in the tumor area. The data are represented as the mean ± SEM ($n = 10–12$ fields, 0.40 mm$^2$ per field) from 3–5 mice in two independent experiments. ***$p<0.001$, (Figure 2—figure supplement 2—source data 1).

DOI: 10.7554/eLife.14756.013

The following source data is available for figure 2:

Figure supplement 2—Source data 1. Density of Tregs and adoptive CTLs in the tumor area.

DOI: 10.7554/eLife.14756.014
Figure 3. Migratory behavior of the adoptive CTLs in the tumor microenvironment of mice treated with CTX-ACT. (A–D) Time-lapse images of CTLs with time-coded motion trajectories (color scale represents the duration). (A,C) Images of CTLs (green) at the periphery (near the blue area, A) or in the parenchyma (in the blue area, C) of the CFP-B16 tumors on different days after CTX-ACT treatment. Scale bar: 100 μm. (B,D) Trajectories of the individual CTLs at the periphery or in the parenchyma were plotted following the alignment of their starting positions. (E) Random walking analysis of the adoptive CTLs. Mean displacement (μm) versus the square root of the time (min^{1/2}) of the CTLs at the periphery of (top) or in the parenchyma (bottom) on different days, (Figure 3—source data 1–9). (F–H) Scatter plots of (F) the mean velocity, (G) the confinement ratio, and (H) the arrest coefficient of the CTLs at the tumor periphery or in the tumor parenchyma on different days after CTX-ACT treatment. Each data point represents a single cell, and the red bars indicate mean values. *p<0.05, **p<0.01, ***p<0.001; ns – not significant, (Figure 3—source data 10). The data from 4–6 mice in three independent experiments were pooled.

DOI: 10.7554/eLife.14756.015

The following source data is available for figure 3:

Source data 1. Mean displacement (μm) versus the square root of the time (min^{1/2}) of the CTLs at the tumor periphery on Day 1. DOI: 10.7554/eLife.14756.016
Source data 2. Mean displacement (μm) versus the square root of the time (min^{1/2}) of the CTLs at the tumor periphery on Day 3. DOI: 10.7554/eLife.14756.017
Source data 3. Mean displacement (μm) versus the square root of the time (min^{1/2}) of the CTLs at the tumor periphery on Day 5. DOI: 10.7554/eLife.14756.018
Source data 4. Mean displacement (μm) versus the square root of the time (min^{1/2}) of the CTLs at the tumor periphery on Day 6. DOI: 10.7554/eLife.14756.019
Source data 5. Mean displacement (μm) versus the square root of the time (min^{1/2}) of the CTLs in the tumor parenchyma on Day 3. DOI: 10.7554/eLife.14756.020
Source data 6. Mean displacement (μm) versus the square root of the time (min^{1/2}) of the CTLs in the tumor parenchyma on Day 5. DOI: 10.7554/eLife.14756.021
Source data 7. Mean displacement (μm) versus the square root of the time (min^{1/2}) of the CTLs in the tumor parenchyma on Day 6. DOI: 10.7554/eLife.14756.022
Source data 8. Linear fitting results of MD (mean displacement) of adoptive CTLs at the tumor periphery on Day 1, Day 3, Day 5 and Day 6. DOI: 10.7554/eLife.14756.023
Source data 9. Linear fitting results of MD (mean displacement) of adoptive CTLs in the tumor parenchyma on Day 3, Day 5 and Day 6. DOI: 10.7554/eLife.14756.024
Source data 10. Scatter plots of the mean velocity, confinement ratio, and arrest coefficient of the adoptive CTLs at the tumor periphery or in the tumor parenchyma on different days. DOI: 10.7554/eLife.14756.025
Figure 4. Migratory behavior of endogenous CTLs in the tumor microenvironment of mice treated with ACT and CTX-ACT on Day 5. (A) In vivo time-lapse images of the endogenous GFP T cells (green) and adoptive CTLs (red) in the CFP-B16 tumor area (blue). Mice were treated with ACT or CTX-ACT. Scale bar: 100 μm. (B) Quantification of endogenous GFP T cells in the differently treated groups on Day 5. Results are represented as the mean ± SEM (n = 11–19 fields, 0.40 mm² per field) from three mice per group. ***p<0.001 (Figure 4—source data 1). (C) Trajectories of GFP T cells in the differently treated groups were plotted following the alignment of their starting positions. (D–F) Scatter plots of (D) the mean velocity, (E) the confinement ratio, and (F) the arrest coefficient of the GFP T cells in tumor areas in the differently treated groups on Day 5. Each data point represents a single cell, and the red bars indicate mean values. *p<0.05, **p<0.01, ***p<0.001; ns: not significant (Figure 4—source data 2). The data from 3–5 mice in two independent experiments were pooled.

DOI: 10.7554/eLife.14756.030

The following source data is available for figure 4:

Source data 1. Quantification of endogenous GFP T cells in the differently treated groups on Day 5.
DOI: 10.7554/eLife.14756.031

Source data 2. Scatter plots of the mean velocity, confinement ratio, and arrest coefficient of the GFP T cells in tumor areas in the differently treated groups on Day 5.
DOI: 10.7554/eLife.14756.032
Figure 4—figure supplement 1. Characterization of GFP cells in differently treated Cxcr6+/gfp mice. (A) Ex vivo analysis and characterization of the GFP cells in the tumors of Cxcr6+/gfp mice that were treated differently. (B) Percentage of GFP cells in the tumors of mice following the different treatments. (C) Percentage of CD8+CD69+ CTLs in the GFP cells from (B).

DOI: 10.7554/eLife.14756.033
**Figure 5.** Migratory behavior of endogenous tumor-infiltrating immunocytes (TIIs) induced by the CTX-ACT treatment. (A) Experimental procedure for long-term intravital imaging of TIIs in the tumor microenvironment. (B) In vivo time-lapse images of EGFP TIIs (green) in the CFP-B16 tumor area (blue) from Day 0 to Day 4 after CTX-ACT treatment. Green arrows represent TIIs displacement, and blue areas represent CFP-B16 tumors. Scale bar: 100 μm. (C) Trajectories of individual TIIs on different days were plotted following the alignment of their starting positions. (D–F) Scatter plots of the (D) mean velocity, (E) confinement ratio, and (F) arrest coefficient of EGFP TIIs in tumor areas on different days. Each data point represents a single cell, and the red bars indicate mean values. *p<0.05, **p<0.01, ***p<0.001; ns, not significant, (*Figure 5—source data 1*). (G) Random walking analysis of the TIIs on different days. Mean displacement (μm) versus the square root of the time (min\(^{1/2}\)) of the TIIs, (*Figure 5—source data 2–7*). The data from 4–7 mice in three independent experiments were pooled.

DOI: 10.7554/eLife.14756.035

The following source data is available for figure 5:

**Source data 1.** Scatter plots of the mean velocity, confinement ratio, and arrest coefficient of EGFP TIIs in tumor areas on different days. DOI: 10.7554/eLife.14756.036

*Figure 5 continued on next page*
Figure 5 continued

**Source data 2.** Mean displacement (µm) versus the square root of the time (min^{1/2}) of the TILs on Day 0.
DOI: 10.7554/eLife.14756.037

**Source data 3.** Mean displacement (µm) versus the square root of the time (min^{1/2}) of the TILs on Day 1.
DOI: 10.7554/eLife.14756.038

**Source data 4.** Mean displacement (µm) versus the square root of the time (min^{1/2}) of the TILs on Day 2.
DOI: 10.7554/eLife.14756.039

**Source data 5.** Mean displacement (µm) versus the square root of the time (min^{1/2}) of the TILs on Day 3.
DOI: 10.7554/eLife.14756.040

**Source data 6.** Mean displacement (µm) versus the square root of the time (min^{1/2}) of the TILs on Day 4.
DOI: 10.7554/eLife.14756.041

**Source data 7.** Linear fitting results of MD (Mean displacement) of TILs at tumor areas on Day 0–Day 4.
DOI: 10.7554/eLife.14756.042
Figure 6. Migratory behavior of the TILs following different treatments. (A) In vivo time-lapse images of the EGFP TILs in the CFP-B16 tumor area on Day 1. Mice were treated with ACT, CTX, CTX-ACT or PBS control. Green arrows represent TILs displacement, and blue areas represent CFP-B16 tumors. Scale bar: 100 μm. (B) The trajectories of individual EGFP TILs in different treated groups were plotted following the alignment of their starting positions. (C–E) Scatter plots of the (C) mean velocity, (D) confinement ratio, and (E) arrest coefficient of the EGFP TILs in the differently treated groups. Each data point represents a single cell, and the red bars indicate mean values. *p<0.05, **p<0.01, ***p<0.001; ns, not significant (Figure 6—source data 1). (F) Random walking analysis of the TILs in the different groups. Mean displacement (μm) versus the square root of time (min^{1/2}) of TILs in different treatment groups (Figure 6—source data 2–6). The data from 12–15 mice in three independent experiments were pooled.

DOI: 10.7554/eLife.14756.044

The following source data is available for figure 6:

**Source data 1.** Scatter plots of the mean velocity, confinement ratio, and arrest coefficient of the EGFP TILs in the different treatment groups.

DOI: 10.7554/eLife.14756.045

**Source data 2.** Mean displacement (μm) versus the square root of the time (min^{1/2}) of the TILs in the PBS group.

DOI: 10.7554/eLife.14756.046

**Source data 3.** Mean displacement (μm) versus the square root of the time (min^{1/2}) of the TILs in the ACT group.

DOI: 10.7554/eLife.14756.047

**Source data 4.** Mean displacement (μm) versus the square root of the time (min^{1/2}) of the TILs in the CTX group.

DOI: 10.7554/eLife.14756.048

**Source data 5.** Mean displacement (μm) versus the square root of the time (min^{1/2}) of the TILs in the CTX-ACT group.

DOI: 10.7554/eLife.14756.049

**Source data 6.** Linear fitting results of MD (Mean displacement) of TILs at tumor areas in the different treatment groups on Day 1.

DOI: 10.7554/eLife.14756.050
Figure 6—figure supplement 1. Phenotype of EGFP TILs in CFP-B16 tumors of mice following different treatments. (A) Representative tumor sections stained with CD3, Ly6G and F4/80. Most of the EGFP TILs at the tumor periphery were Ly6G⁺ and F4/80⁺. Scale bar: 50 μm. (B) Histopathology of HE-stained tumor sections from tumor-bearing mice exposed to different treatments. Black arrows indicate neutrophils. Scale bar: 50 μm. (C) Percentage of neutrophils in different treatment groups.
neutrophils among TILs at the periphery of the tumors in mice that received different treatments. The data are represented as the mean ± SEM (n = 14–20 fields) results from three mice per group. *p<0.05, **p<0.01, (Figure 6—figure supplement 1—source data 1).

**Figure supplement 1—Source data 1.** Percentage of neutrophils among TILs at the periphery of the tumors in mice that received different treatments. DOI: 10.7554/eLife.14756.052
Figure 7. Intravital imaging of DCs infiltrating into the tumor areas of the mice following different treatments. (A) Large-field intravital imaging of DCs (green) and Tregs (red) in the CFP-B16 tumor area (blue) on Day 3. Top row: large-field images; scale bar 500 µm. Bottom row: images of the region of interest from the top row; scale bar 100 µm. (B) Density of DCs in the tumor areas in the different treatment groups. The data are represented as the mean ± SEM (n = 10–14 fields, 12 mm² per field [large-field images] or 0.40 mm² per field) from three independent experiments. *p<0.05, **p<0.01, ***p<0.001; ns, not significant (Figure 7—source data 1). (C) Representative images of mature DCs in tumor sections were immunofluorescently labeled to detect MHC II (top row) and CD86 (bottom row). Scale bar: 40 µm. Inserts are magnifications of the regions indicated with arrows. Scale bar: 15 µm.

DOI: 10.7554/eLife.14756.054

The following source data is available for figure 7:

Source data 1. Density of DCs in the tumor areas in the different treatment groups.
DOI: 10.7554/eLife.14756.055
Figure 8. Metronomic CTX-ACT therapy efficiently controlled the growth of CFP-B16 tumors in vivo. (A) Metronomic therapy schedule of the CTX-ACT treatment. (B) Growth curves for CFP-B16 tumors in the different treatment groups. The data are represented as the mean ± SEM tumor volume (n = 12–15, three independent experiments). *p<0.05, **p<0.01, ***p<0.001(Figure 8—source data 1). (C) Long-term and large-field intravital images of the tumor microenvironment during CTX-ACT metronomic therapy. Blue, a CFP-B16 tumor; green, EGFP host cells. Scale bar: 500 μm.
DOI: 10.7554/eLife.14756.056
The following source data is available for figure 8:
Source data 1. Growth curves for CFP-B16 tumors in the different treatment groups
DOI: 10.7554/eLife.14756.057
Figure 9. Timeline schematic showing the elicitation of anti-tumor immune responses in the tumor microenvironment by CTX-ACT treatment. Step 1: CFP-B16 tumor cells grew and Tregs accumulated in the tumor area before CTX treatment. Step 2: CTX treatment depleted most Tregs, blocking the formation of an ‘immunosuppressive ring’. Step 3: A transient increase in the motility of the endogenous neutrophils is elicited by CTX-ACT treatment on Day 1. Step 4: DCs present increased infiltration on Day 3. Step 5: Adoptive CTLs present increased infiltration and motility and the CTX-ACT treatment retained activated endogenous CTLs in the tumor area on Day 5. Step 6: Solid tumor ‘melting’ occurs from the inside and ‘shrinking’ occurs from the outside on Days 5–6.

DOI: 10.7554/eLife.14756.058