In situ Immunogenic Clearance Induced by a Combination of Photodynamic Therapy and Rho-kinase Inhibition Sensitizes Immune Checkpoint Blockade Response to Elicit Systemic Antitumor Immunity against Intraocular Melanoma and its Metastasis

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Supplementary materials:
Graphical Abstracts, Supplementary methods and figures.
Graphical Abstracts

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Combination of localized photodynamic therapy with FIC, and rho-kinase inhibitor exerts a cancer vaccine-like function. Immunogenic clearance led to the trafficking of CD8$^+$ T cells into primary tumor site, sensitized immune checkpoint blockade response. Eventually, combined treatment evoked systemic anti-tumor immunity to inhibit metastasis, one of the major issues facing uveal melanoma therapy.
Supplementary methods

Synthesis of FIC

FIC was prepared as reported in our previous study.[14] Briefly, a solution of Pluronic F-127 (20 mg, Aldrich, St. Louis, MO, USA) as a surfactant, diatrizoic acid (2 mg, Aldrich) as an aromatic matrix, and Ce6 (10 µg/mL, Frontier Scientific, Logan, UT, USA) was prepared in 1 mL methanol and the solvent was slowly evaporated by heating under reflux conditions. The completely dried hot melt was quenched in an ice bath and mixed with distilled water (2 mL) to disperse self-assembled Pluronic-based nanoparticles by vigorous shaking.

Cell viability assay

Cell viability assays were conducted using Cell Counting Kit-8 (Donjindo Molecular Technologies, Kumamoto, Japan). For CCK-8 assays, B16F10 cells were seeded and incubated overnight. At 24 h after PDT treatment, CCK-8 solution was added to each well, and the plates were incubated for 2 h according to the manufacturer’s instructions; optical absorbance was measured at 450 nm.

In vitro CRT translocation to cell surface

For confocal microscopy, B16F10 cells were seeded into 4-well chamber slides at 4 × 10⁴ cells/well and incubated in media overnight. After PDT treatment and 1 h of further incubation, the cells were washed with PBS and fixed with 0.25% paraformaldehyde for 5 min. The cells were washed again with PBS and incubated in 3% bovine serum albumin containing PBS blocking buffer for 30 min at 4°C. These cells were incubated with anti-
calreticulin primary antibody and Alexa Fluor 488 anti-rabbit secondary antibody. Cell nuclei were stained with Hoechst 33342. Images were obtained by confocal laser scanning microscopy.

**Multiplex IHC image acquisition and analysis**

Slides were scanned using the Vectra 3.0 Automated Quantitative Pathology Imaging System (Perkin-Elmer), and images analysis was performed using the InForm 2.2 software and TIBCO Spotfire™ (Perkin-Elmer). Individual extracted images of sections were stained with Ki-67-Opal 570, F4/80-Opal 520, PD-L1-Opal 690, FOXP3-Opal 620, CD4-Opal 480, CD8-Opal 780, and DAPI were used to establish a spectral library of fluorophores required for multispectral analysis. Using this spectral library, we formed a reference for target quantitation, as the intensity of each fluorescent target was extracted from the multispectral data using linear unmixing. Each cell was identified by detecting nuclear spectral elements (DAPI). Immune phenotypes of cells and populations of them were characterized and quantified using the InForm image analysis software and used as input for the Spotfire™ software; the threshold for positivity of each factor was determined based on IHC scoring methods. All cells were designated as positive or negative for each antibody based on set-up cut-off values and expression intensities were compared.

**Immunohistochemistry analysis**

Formalin-fixed paraffin-embedded tissue blocks were cut into 4-μm-thick sections. Sections were heated at 60°C for at least one hour and deparaffinized with xylene, followed by multiplex immunofluorescence staining with a Leica Bond Rx™ Automated Stainer (Leica
Biosystems, Newcastle, UK). Briefly, all slides were baked for 30 mins, dewaxed with Leica Bond Dewax solution (#AR9222, Leica Biosystems) and washed with Bond Epitope Retrieval 2 (#AR9640, Leica Biosystems) in a pH 9.0 solution for 30 mins for antigen retrieval.
Supplementary figures

**Figure S1. Analysis of cell death induction and detection of hallmarks of immunogenic cell death triggered by FIC-PDT in B16F10 cells.** A) The viability of B16F10 cells after FIC incubation with different concentration with or without LED irradiation. B) CLSM images of CRT translocation to membrane of B16F10 cells upon FIC-PDT treatment.
Figure S2. Analysis of cell death induction and detection of hallmarks of immunogenic cell death triggered by FIC-PDT in 92.1 cells. A) The viability of 92.1 cells after FIC incubation with different concentration with or without LED irradiation. B) CLSM and flow cytometric analyses of CRT translocation to the 92.1 cell surface. (Left: CLSM images, Right: low-cytometric histograms) Data presented as relative MFI. C) Immunoblotting of HMGB1 and HSP70 released in conditioned media after FIC-PDT treatment or not. One-way ANOVA test was used for statistics; *p < 0.05, **p < 0.01, ***p <0.001. Data are presented as mean ± SD.
Figure S3. Analysis of cell death induction and detection of hallmarks of immunogenic cell death triggered by FIC-PDT in CT26 cells. A) Dying or dead CT26 cells after FIC-PDT treatment on varying concentration of FIC. The percentage represents early (PI negative) and late (PI positive) apoptosis. B) Flow cytometric analyses of CRT expression on the surface of cell membrane gated in PI intact cells. Data presented as relative MFI. C) Immunoblotting of HMGB1 and HSP70 released in conditioned media after FIC-PDT treatment or not. D) Quantification of secreted ATP from FIC-PDT treated B16F10 cells. One-way ANOVA followed by Tukey’s post hoc test was used for statistics; *p < 0.05, **p < 0.01, ***p <0.001. Data are presented as mean ± SD.
Figure S4. BMDMs and BMDCs differentiation and quantification of phagocytic activity of phagocytes

A) Differentiation of BMDMs and BMDCs from mice bone marrow was estimated by flow cytometry. B) Quantification of immunofluorescence images of engulfed non-treated or FIC-PDT treated (0.75 μg/ml, 65 mW/cm²) B16F10 cells by BMDMs and BMDCs upon non-treated or treated with ripasudil (30 μm). One-way ANOVA test was used for statistics; *p < 0.05, **p < 0.01, ***p < 0.001. Data are presented as mean ± SD.
Figure S5. Phagocytic activity of BMDCs against 92.1 cells A) Quantification of immunofluorescence images of engulfed non-treated or FIC-PDT treated (0.75 μg/ml, 65 mW/cm²) 92.1 cells by BMDCs upon non-treated or treated with ripasudil (30 μm). One-way ANOVA test was used for statistics; *p < 0.05, **p < 0.01, ***p < 0.001. Data are presented as mean ± SD.
Figure S6. Toxicity analysis in vivo. A) Body weight changes of B16F10 bearing mice over 21 days after various treatments. (n=10-13 per group) B, C) FIC nanoparticle alone was I.T injected or LED was solely irradiated on day 6 and 8. B) Tumor growth curves of B16F10 tumors and C) body weight changes of B16F10 bearing mice. (n=6-9 per group) One-way ANOVA followed by Tukey's post hoc test was used for statistics; n.s., not significant. Data are presented as mean ± S.E.M.
Figure S7. Anti-tumor immune response in vivo after combined treatment of FIC-PDT and ripasudil. A) In vivo immunogenic cell death induction upon FIC-PDT treatment. B) Flow cytometric analysis of DC maturation by the costimulatory molecules CD40 and CD86 gated in CD11c+ cells from TDLNs. The percentage (left) and value of MFI (right). C) CLSM examination of hallmarks of immunogenic cell death (CRT, HMGB1 and HSP70) in excised tumors from B16F10 mice after FIC-PDT treatment. D) Representative dot plots of CD8+ T cells (CD8+CD3+ cells) gated in CD45.2+ cells from excised tumor by flow cytometry. One-way ANOVA followed by Tukey’s post hoc test was used for statistics; *p < 0.05, **p < 0.01, ***p < 0.001. Data are presented as mean ± S.E.M.
Figure S5. In vivo PD-L1 expression in TME. A, B) PD-L1 expression in TME is assessed by flow cytometry (A) and CLSM (B). (n=3 per group) One-way ANOVA followed by Tukey’s post hoc test was used for statistics; *p < 0.05, **p < 0.01, ***p < 0.001. Data are presented as mean ± S.E.M.
Figure S6. Abscopal effect of local administration of FIC-PDT and ripasudil combined with α-PD-L1 antibody. A) Individual tumor growth curves of each group (n=8-12 per group) B-C) Percentage of (B) Ki-67 and (C) IFN-γ expressing CD8+ T cells in primary tumor was quantified by flow cytometry. D) Percentage of regulatory (CD25+CD4+) T cells in primary and secondary tumors was quantified by flow cytometry. One-way ANOVA followed by Tukey's post hoc test was used for statistics; *p < 0.05, **p < 0.01, ***p < 0.001. Data are presented as mean ± S.E.M.
Figure S7 Immunogenic cell death induction by FIC-PDT with laser. A) Cell viability of B16F10 cells after FIC incubation with various concentration with or without laser irradiation, (300 mW/cm², 1 min) B) Immunoblotting of HSP70 released in conditioned media after FIC-PDT treated or not.
Figure S8. Anti-tumor effect in orthotopic intraocular melanoma model. A) Treatment schedule for UM orthotopic model. B) Representative images of mice upon Ripa + PDT + α-PD-L1 treatment. C) Histologic appearance of tumor upon each treatment was investigated by hematoxylin and eosin (H&E) staining.
Figure S9. Population and location of immune cell subsets in tumor using multiplex IHC. A) Tumor tissues were stained with Ki67 (yellow), PD-L1 (red), FOXP3 (orange), CD8 (white) and nuclei was co-stained with DAPI (blue). B-D) Immune cell subsets were quantified as each immune cell numbers in total cells in TME. B) Percentage of CD8+ cells, C) percentage of proliferative CD8+ (Ki67+CD8+) cells and D) the ratio of CD8+ cells to FOXP3+ cells.