Abstract

Although PD-1 blockade cancer immunotherapy has shown potential for a wide range of patients with cancer, its efficacy is limited, in part, due to the loss of effector cytotoxic T lymphocytes (CTLs) via terminal differentiation–induced apoptosis. We previously demonstrated that mitochondrial activation, by the agonists of peroxisome proliferator–activated receptor γ (PPARγ) coactivator 1-α (PGC-1α)/transcription factor complexes, had synergistic effects with a PD-1–blocking monoclonal antibody in a mouse tumor model. In the current study, we examined the molecular mechanism of the synergistic effects of bezafibrate, an agonist of PGC-1α/ PPAR complexes, which enhanced the tumoricidal effects of PD-1 blockade. Bezafibrate activated CTL mitochondria and upregulated oxidative phosphorylation as well as glycolysis, resulting in more proliferation of naïve T cells and improved effector function in CTLs. Bezafibrate also increased fatty acid oxidation (FAO) and mitochondrial respiratory capacity, which supports the extra energy demands of cells in emergencies, allowing cell survival. Camitine palmitoyl transferase 1 (Cpt1), which is needed for FAO, and Bcl2 were both upregulated. Cpt1 and Bcl2 can form a complex to prevent apoptosis of CTLs. Together, these results indicate that bezafibrate increases or maintains the number of functional CTLs by activating mitochondrial and cellular metabolism, leading in turn to enhanced antitumor immunity during PD-1 blockade. Cancer Immunol Res; 6(11); 1375–87. ©2018 AACR.
that PGC-1α, a regulator of mitochondrial biogenesis, is important for development of T-cell-mediated antitumor immunity (21). We demonstrated previously that activation of PGC-1α/peroxisome proliferator-activated receptors (PPAR) by bezafibrate improves the efficacy of PD-1 blockade (20). However, the mechanism by which the PPAR pathway activates antitumor immunity remains unknown.

In this study, we investigated the effect of bezafibrate on the phenotype of effector CD8+ T cells and on mitochondria. We investigated the molecular mechanism by which bezafibrate modulates CTL differentiation and enhances T-cell–based antitumor immunity. We found that bezafibrate promotes differentiation of naïve to effector T cells, upregulates FAO, and inhibits apoptosis of effector T cells. These different effects of bezafibrate on the priming and effector phases resulted in increased numbers of functional effector T cells and improved effectiveness of PD-1 blockade. Therefore, combining the increased numbers of functional effector T cells and improved immunity remains unknown.

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Materials and Methods

Mice and cells

C57BL/6N and BALB/c mice were purchased from The Charles River Laboratory. CD8−/− mice were provided from The Jackson Laboratory (originally from Univ. Toronto, T.W. Mak). Mice were approximately 5 to 6 weeks old at the time of the experiment and maintained under specific pathogen-free conditions at the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, under the direction of the institutional review board. The murine fibrosarcoma cell line (MethA) was obtained from the Cell Resource Center for Biomedical Research, and murine colon carcinoma cell line (MC38) was a gift from Dr. James P. Allison, Memorial Sloan-Kettering Cancer Center (New York, NY). Cell lines were cultured in RPMI medium (Gibco; 11875-093) with 10% fetal bovine serum and 1% penicillin-streptomycin mixture solution (Nacalai Tesque; 26253-84). Cell lines were free of mycoplasma contamination. Cell lines were used within fifth passage and were not authenticated in the past year.

Mouse therapy model

A total of 5 × 105 cells of MC38 and MethA were intradermally (i.d.) injected into the right flank of C57BL/6N and BALB/c mice, respectively (day 0). Combination therapy was started when the tumor size reached 60 to 70 mm3 (around day 7). Mice were intraperitoneally (i.p.) injected with 40 μg of anti-PD-L1 (clone 1-111A.4) and mAb injection was repeated every sixth day. Bezafibrate (Santa Cruz Biotechnology) was i.p. injected at the concentration of 30 mg/kg every third day. For untreated mice, an isotype control (flank of C57BL/6N and BLAB/c mice, 2.5 mg/kg every third day. For untreated mice, an isotype control (Cpt1a (8F6AE9) from Abcam. All flow cytometry experiments were performed on a FACSCant o II (BD Biosciences) and with recombinant human IL2 (100 U/mL; PeproTech). The cell preparation for analysis

Cell lines were used within fifth passage and were not authenticated in the past year.

CD8+ T cells were isolated from the lymph node and spleen of CD45.1 congenic mice using an autoMACS Pro Separator (Miltenyi Biotech). After washing with PBS, CD8+ T cells were labeled with CellTrace Violet (Thermo Fisher Scientific). For labeling, CD8+ T cells were incubated for 15 minutes with CellTrace diluted in PBS. After quenching with complete media and washing the cells twice with PBS, the labeled CD8+ CD45.1+ T cells were intravenously (i.v.) injected in to CD45.2 CD8−/− mice. MC38 cells (5 × 105 cells) were i.d. injected 2 days after CD8+ T-cell infusion. Anti–PD-L1 and bezafibrate were i.p. administered 5 days after tumor inoculation. Mice were sacrificed on day 9 for analysis.

Chemical reagents

Bezafibrate was used at the dose of 2.5 mg/kg for combination therapy. Bezafibrate was freshly prepared immediately before use in DMSO. Dissolved bezafibrate was diluted in PBS and 200 μL was i.p. injected per mouse. Bezafibrate was added at the concentration of 30 μmol/L for the entire culture period.

Naïve CD8+ T-cell sorting using AutoMACS

To isolate naïve CD8+ T cells from C57BL/6N wild-type mice, the spleen and three LNs (axillary, brachial, and inguinal LNs) from both the right and left sides were harvested. The spleen was minced, treated with ACK buffer for 2 minutes to lyse erythrocytes, and mixed with pooled and minced LN cells. naïve (CD44−) CD8+ T cells were then purified from total pooled lymphocytes according to the manufacturer's instructions (Miltenyi Biotec; 130-096-543).

Cell preparation for analysis

For draining lymph node (DLN) analysis, axillary, brachial, and inguinal LNs (one of each) were harvested from the right side of tumor-bearing mice. All LNs were minced and pooled. Average LN cell numbers (total pooled LN cells/3) were used as absolute cell numbers. For tumor-infiltrating lymphocyte (TIL) analysis, tumor tissue was harvested and minced into 1- to 2-mm pieces with scissors followed by digestion with collagenase type IV (Worthington Biochemical Corporation) using a gentleMACS Dissociator (Miltenyi Biotec). The numbers of TILs per mg of tumor tissue were used as the absolute numbers. For in vitro analysis, naïve CD8+ T cells were stimulated with beads coated with anti-CD3 and CD28 (Thermo Fisher Scientific) and with recombinant human IL2 (100 U/mL; PeproTech). The expanded T cells on days 2 and 13 were used for proliferation assays and apoptosis assays, respectively.

Flow cytometry analysis

The following monoclonal antibodies (mAb) were used to detect the respective antigens: CD44 (IM7), CD45.2 (104), CD45.1 (A20), CD8 (53-6.7), CD62L (MEL-14), T-bet (4B10), IFNγ (XMG-1.2), and Bcl-2 (BCL1/10C4) from BioLegend; EOMES (Dan11mag), and Ki67 (SolA15) from ebioscience: Cpt1a (8F6AE9) from Abcam. All flow cytometry experiments were performed on a FACS Canto II (BD Biosciences) and analyzed using FlowJo software (FlowJo, LLC). Details of intracellular staining were mentioned previously (20). Mitochondrial mass, mitochondrial potential, mitochondrial superoxide, and cellular reactive oxygen species (ROS) were determined by MitoTracker Green, MitoTracker Deep Red, MitoSOX Red, and CellROX Green reagents, respectively (all from Life Technologies). These dyes were added to the cells at final concentrations of 0.125, 0.125, 5.0, and 0.625 μmol/L and incubated at 37°C in a 5% CO2 humidified incubator for 30 minutes, followed by surface staining.
Measurement of oxygen consumption rates and extracellular acidification rate

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of CD8\(^+\) T cells isolated from treated mice were measured using an XF\(^96\) Extracellular Flux analyzer (Seahorse Biosciences). Cells (400,000 per well) were seeded in an XF\(^96\) plate, determined by titration, as previously described (20). Different parameters from the OCR graph were calculated. ATP turnover was defined as follows: (last rate measurement before oligomycin) – (minimum rate measurement after oligomycin injection). Maximal respiration was defined as follows: (maximum rate measurement after FCCP) – (nonmitochondrial respiration). Spare respiratory capacity (SRC) was calculated by subtracting basal respiration from maximal respiration. We measured the ECAR value in the same well, which contained an optimal glucose level so the basal ECAR (or glycolysis) value is the reading we obtained immediately before oligomycin injection. We prepared the assay medium as described in the XF cell Mito Stress Test Kit (Kit 103015-100). The glucose concentration in this medium is 10 mmol/L. In the classic glycolytic assay procedure (glucose-free media), the final concentration of glucose added to the port is 10 mmol/L while measuring flux. The basal ECAR value in this method is calculated by subtracting the last rate measurement before glucose injection from the maximum rate measurement before oligomycin injection, which gives the same value as calculated by our method. Glycolytic capacity was defined as the rate measured after oligomycin injection. Glycolytic reserve was defined as follows: (glycolytic capacity) – (basal ECAR value) (22).

Real-time RT-PCR

We isolated RNA from purified CD8\(^+\) T cells with the RNeasy mini kit (QIAGEN) and synthesized cDNA by reverse transcription (Invitrogen). The primers used to perform real-time PCR are listed in Supplementary Table S1.

Microarray analysis

Total RNA was extracted using the RNeasy Micro kit (QIAGEN) according to the manufacturer’s protocols. CD8\(^+\) T cells expanded in vitro until day 13 were lysed according to the protocol. Microarray analysis was performed by Macrogen using GeneChip Mouse Gene 2.0 ST Array. The data were deposited at GEO repository (http://www.ncbi.nlm.nih.gov/geo) and the accession number is GSE118659. Gene-enrichment and functional annotation analysis was performed using Gene Ontology (www.genontology.org) and KEGG (www.genome.jp/kegg/). All data analysis and visualization of differentially expressed genes was conducted using R 3.1.2 (www.r-project.org).

Western blotting

CD8\(^+\) T cells were isolated from DLNs using mouse CD8 MicroBeads (Miltenyi Biotec). After washing the cells with PBS twice, 2 \times 10^5 cells were used. Details were as described (20). Antibodies recognizing p-CREB (ab32096) and Cpt1a (ab128568) were obtained from Abcam.

Statistical analysis

Statistical analysis was performed using Prism 6 (GraphPad Software). One-way ANOVA analysis followed by Sidak multiple comparison test was utilized to analyze three or more variables. To compare two groups, Student t test was used. All statistical tests were two-sided assuming parametric data, and a P of <0.05 was considered significant. The variations of data were evaluated as the means ± standard error of the mean (SEM). Five or more samples were thought to be appropriate for the sample size estimate in this study. Samples and animals were randomly chosen from the pool and treated. Treatment of samples and animals was performed unblinded.

Results

Bezafibrate expands CD8\(^+\) T cells and enhances effector function of CTLs

In our previous study, we demonstrated mitochondrial activation during PD-1 blockade therapy and developed several combination therapies using mitochondria-activating chemicals. We found that activation of the PGC-1α/PPARs axis by bezafibrate improved the efficacy of PD-1 blockade in murine colon carcinoma (MC38)-bearing C57BL/6 mice (20). We confirmed similar synergistic effects of bezafibrate with anti-PD-L1 on the growth of MethA tumors, a murine skin sarcoma line on a different genetic background, BALB/c (Supplementary Fig. S1A). Bezafibrate treatment alone did not exhibit any antitumor activity, indicating that enhanced antitumor activity is mediated through activated lymphocytes, but not directly through tumor cells (ref. 20; Supplementary Fig. S1B). These results indicate that the combination of bezafibrate with PD-1 blockade is applicable to multiple tumors on different genetic backgrounds.

Because the number of effector killer T cells determines the effect against cancer cells in vivo, we first investigated the effect of bezafibrate on the effector phenotype of CD8\(^+\) T cells (15–17). Both the frequency and number of effector/memory CD8\(^+\) T cells (CD62L\(^−\)CD44\(^+\) CD8\(^+\) T cells, P3) in DLNs were significantly increased by treatment combining anti-PD-L1 and bezafibrate compared with treatment with anti-PD-L1 alone (Fig. 1A). In contrast, the numbers of naive (CD62L\(^+\)CD44\(^−\) CD8\(^+\) T cells, P1) and central memory T cells (CD62L\(^+\)CD44\(^−\) CD8\(^+\) T cells, P2) were not changed by bezafibrate addition, whereas injection of anti-PD-L1 alone was accompanied by increased P1 and P2 populations (Fig. 1A). Accordingly, CD8\(^+\) TILs, which mainly comprise the effector/memory T-cell population (P3), were expanded, as we previously described (ref. 20; Fig. 1B).

We attribute the antitumor effect not only to the number of effector T cells but also to their function. The T-bet/Eomes balance is a factor regulating cytokine synthesis and T-cell differentiation (22). We found that bezafibrate addition increased the amount of T-bet in DLN CD8\(^+\) T cells (Fig. 1C). In contrast, the frequency and number of Eomes\(^+\) CD8\(^+\) T cells decreased or were unchanged after bezafibrate addition (Fig. 1C). These data demonstrate that the bezafibrate enhanced the effector function of CTLs. We further investigated T-bet and Eomes expression in each P1–P3 population. Among subsets of T cells, the P3 population has the most T-bet and the least Eomes; T-bet increased after bezafibrate treatment only in P3 (Supplementary Fig. S2A). Although the naive population (P1) is present most abundantly in CD8\(^+\) T cells, T-bet expression is lowest in this population and is unaffected by bezafibrate treatment (Supplementary Fig. S2A). Bezafibrate combination therapy increased the number of cells only in the P3 population (Fig. 1A). These results suggest that most of the changes in T-bet amounts are due to the P3 population in DLN CD8\(^+\) T cells (Fig. 1C). CD8\(^+\) TILs are T-bet\(^+\) and Eomes\(^+\).
Supplementary Fig. S2B) and exclusively comprised of P3 population (Fig. 1B). These data suggest that bezafibrate treatment enhances the function of effector/memory CD8\(^+\) T cells. Indeed, IFN\(\gamma\) in both CD8\(^+\) T cells of DLN and CD8\(^+\) TILs was upregulated (Supplementary Fig. S2C; Fig. 1D). Together, we showed the bezafibrate combination therapy increases the number and enhances the function of effector/memory CD8\(^+\) T cells in DLNs and at the tumor site.

Figure 1.
Bezafibrate improves the quality and quantity of effector CD8\(^+\) T cells in vivo. MC38-bearing mice were treated with anti–PD-L1 and bezafibrate on the same schedule as shown in Supplementary Fig. S1A. Mice were sacrificed, and CD8\(^+\) T cells in DLN and tumor sites were analyzed on the indicated day. A, DLN cells on day 15 were stained with anti–CD8, anti–CD62L, and anti–CD44. Representative FACS patterns are shown (top). The absolute numbers of P1–P3 population per one LN were calculated (bottom). B, Cells isolated from the tumor mass on day 11 were stained with anti–CD8, anti–CD45.2, anti–CD62L, and anti–CD44. CD45.2\(^+\) CD8\(^+\) T cells were gated, and representative FACS patterns are shown (left). The frequencies and number of CD45.2\(^+\) CD8\(^+\) TILs were compared between treated groups (middle). CD62L and CD44 phenotypes after gating at CD45.2\(^+\) CD8\(^+\) T cells were analyzed (right). C, T-bet and Eomes expression was analyzed by flow cytometry in DLN CD8\(^+\) T cells on day 15 from treated mice. Representative FACS data are shown (left). The frequency and numbers of T-bet\(^+\) or Eomes\(^+\) T cells were calculated in DLN CD8\(^+\) T cells from treated mice (right). D, Digested tumor tissues on day 15 were incubated at 37°C for 6 hours, and IFN\(\gamma\) was intracellularly stained in CD8\(^+\) T cells from treated mice. Representative FACS data of CD8\(^+\) T cells gated (left), frequency (middle), and MFI (right) of IFN\(\gamma\)^+ T cells among CD8\(^+\) T cells are shown.

A–D, Data represent the means ± SEM of five mice. Data are representative of two independent experiments. *\(P<0.05; **, P<0.01\), one-way ANOVA analysis.
Beazafibrate with PD-1 blockade induces mitochondrial activation in CD8+ T cells

To understand how beazafibrate enhances T-cell–mediated antitumor immunity under PD-1 blockade conditions, we investigated mitochondrial activities in CTLs isolated from DLNs of MC38 tumor-bearing mice treated with beazafibrate and anti–PD-L1. We found that the OCR, an indicator of mitochondrial respiration including basal respiration, as well as maximal respiration and ATP turnover were all significantly higher in CD8+ T cells isolated from DLNs of beazafibrate- and anti–PD-L1-treated mice (Fig. 2A, left; Supplementary Fig. S3A–S3B). SRC, which is calculated by subtracting basal respiration from maximal respiration, of CD8+ T cells isolated from DLNs was significantly higher in the combination-therapy group than after PD-1 blockade alone (Fig. 2A, right). Because SRC has been linked to cell survival, enhancement of SRC by beazafibrate and anti–PD-L1 combination therapy suggests that CD8+ T cells can survive longer (23). We also determined the effect of beazafibrate treatment on the ECAR to measure glycolysis. We observed that the ECAR values in the beazafibrate and anti–PD-L1 combination-therapy group were significantly higher than those in the group treated with anti–PD-L1 alone (Fig. 2B). The higher values for both OCR and ECAR indicated that CTLs were in a metabolically higher state in the beazafibrate combination group (Fig. 2B). The ratio of OCR and ECAR was higher in the beazafibrate combination group than in the group treated with PD-1 blockade alone (Fig. 2C), indicating that beazafibrate combination therapy raised CD8+ T cells to a higher energy state through mechanisms that relied more on mitochondrial metabolism than on glycolysis. Similar results were observed when the mice were sacrificed at a different time point (Supplementary Fig. S3C and S3D).

As the beazafibrate and PD-1 blockade combination enhanced the OCR values compared with PD-1 blockade alone, we investigated the effect of this combination on other mitochondrial activation parameters. The effector/memory population of CD8+ T cells (P3) in any treatment group showed larger mitochondrial areas, higher intensity of MitoTracker DeepRed, and more ROS than either the naïve (P1) or central memory (P2) CD8+ T cells (Fig. 2D and E). Cellular levels of the MitoTracker DeepRed and ROS increased when cells were treated with beazafibrate combined with anti–PD-L1 (Fig. 2E). Similar results were obtained from CD8- TILs (Fig. 2F). PD-1 blockade significantly reduced various mitochondrial activation parameters in the P3 population as well as in TIL compared with untreated cells (Fig. 2E and F). This reduction may reflect the change of dependency on the energy metabolic pathways from OXPHOS to glycolysis by PD-1 blockade. Indeed, monotherapy enhanced glycolysis-dependent energy production more than OXPHOS (Fig. 2B and C) and (19, 24). In total, beazafibrate and PD-1 blockade combination treatment activated mitochondria in CTLs and increased mitochondrial SRC, contributing to the enhanced survival of CTLs in this group.

Combination therapy enhances mitochondrial biogenesis and FAO in T cells

Because combined beazafibrate and anti–PD-L1 treatment enhanced mitochondrial activities in CD8+ T cells in MC38 tumor-bearing mice, we used qPCR analysis to ask whether the combination therapy affected transcription of genes involved in mitochondrial biogenesis. We detected increased transcription of PGC-1α and transcription factor A–mitochondrial (TFAM), both of which regulate mitochondrial biogenesis. Transcription of other mitochondria-associated genes such as Ubiquinol-Cytochrome C Reductase Core Protein I (Uqrc1), NADH:ubiquinone oxidoreductase core subunit S8 (NDUSF8), and ATP synthase F1 subunit alpha (ATP5a1) also increased in CD8+ T cells from DLNs of mice treated with the combined beazafibrate and PD-1 blockade (ref. 25; Fig. 3A). Because PPAR signaling also activates the FAO pathway, we assessed transcription of enzymes involved in FAO (26). As shown in Fig. 3B, the expression of carnitine palmitoyl transferase 1B (Cpt1b), acyl-CoA dehydrogenase long chain (LCAD), and acyl-CoA dehydrogenase medium chain (MCAD) was significantly increased in CD8+ T cells isolated from DLNs after the combination treatment compared with cells treated with PD-1 blockade alone. We observed that expression of the Cpt1a protein, another FAO enzyme, was increased significantly by beazafibrate combination treatment in CD8+ T cells of both DLN and TIL (Supplementary Fig. S4A and B). Thus, the beazafibrate and PD-1 blockade combination activates mitochondrial biogenesis and FAO in CD8+ T cells in vivo.

Combination therapy enhances survival and proliferation of tumor-reactive CTLs

To investigate the effect of combination therapy on tumor-reactive CTLs, we followed our previous strategy to identify the tumor-reactive CTLs in a mouse tumor model (20). Accordingly, CellTrace-labeled CD45.1+CD8+ T cells were transferred into CD45.2+ mice and their proliferation in DLNs and tumor sites was examined (Fig. 4A). As tumors grow faster in CD8-/- mice than in wild-type mice, tumor reached to the desired volume for start of the therapy earlier (on day 5) in CD8-/- mice than in wild-type mice (on day 7). Among the transferred CD45.1+CD8+ T cells, we identified the proliferating cell population as tumor-reactive cells in mice bearing MC38 tumor cells (Fig. 4B). The frequency and number of proliferating CD45.1+CD8+ T cells were significantly increased in DLNs and at tumor sites of tumor-bearing mice treated with beazafibrate and anti–PD-L1 compared with mice injected with anti–PD-L1 alone (Fig. 4B). The beazafibrate and anti–PD-L1 combination therapy enhanced mitochondrial mass, MitoTracker DeepRed, mitochondrial ROS, and cellular ROS in tumor-reactive CTLs of DLN (Supplementary Fig. S5A).

An increased number of tumor-reactive CTLs enhances antitumor activity in PD-1 blockade therapy, as most PD-1–blocked CTLs undergo terminal differentiation and apoptosis (27). The increase in the number of tumor-reactive CTLs driven by beazafibrate treatment may be caused in two ways: (1) beazafibrate inhibited terminal differentiation and apoptosis of effector T cells or (ii) beazafibrate promoted proliferation associated with the transition from naïve to effector T cells. To test the first possibility, we analyzed apoptotic effector T cells in tumor-reactive CTLs by co-staining with annexin V and propidium iodide (PI). We found that combination therapy significantly reduced the percentage of apoptotic cells (annexin V+ PI- cells) in tumor-reactive CTLs compared with therapy with anti–PD-L1 alone, indicating that beazafibrate addition enhanced survival of tumor-reactive CTLs (Fig. 4C). We also used annexin V and PI staining to analyze the non–tumor-reactive cell population (CellTracehighCD8+ T cells), which includes more live cells than found among the tumor-reactive population (Supplementary Fig. S5B). These observations are consistent with previous reports that showed that upon PD-1 blockade, dysfunctional effector cells regain effector function but die by terminal differentiation (27). We found that the
Bezaflibrate combination therapy enhances activation of mitochondria in killer T cells. MC38-bearing mice were treated with anti–PD-L1 and bezafibrate on the same schedule as shown in Supplementary Fig. S1A. On day 9, the mice were sacrificed and CD8$^+$ T cells in DLN and tumor sites were analyzed. A, OCR of DLN CD8$^+$ T cells isolated from each group was measured. Cells were pooled from five mice. SRC were calculated. 

B, ECAR of the same cells used in A was measured and basal ECAR was calculated. Basal OCR and ECAR values from all treated groups are plotted. 

C, OCR/ECAR ratio was measured. 

D, DLN cells were stained with anti-CD8, anti-CD62L, and anti-CD44. Representative FACS profiles of P1–P3 stained with the indicated mitochondrial dyes in the mice treated with anti–PD-L1 and bezafibrate are shown. E, Representative FACS profiles of P3 population stained with the indicated mitochondrial dyes in each group are shown (top). MFI of P1–P3 stained with each dye was compared between treated groups (bottom). Colors correspond to those of the P1–P3 populations. F, Cells isolated from the tumor mass were stained with anti-CD8 and anti-CD45.2. CD45.2$^+$ CD8$^+$ T cells were gated (top left). Representative FACS profiles of CD45.2$^+$ CD8$^+$ T cells stained with each mitochondrial dye in mice treated with anti–PD-L1 and bezafibrate (top right). MFI of CD45.2$^+$ CD8$^+$ T cells stained with each dye were compared between treated groups (bottom). A–C, Data represent the means ± SEM of 6 wells. Data are representative of two independent experiments. * P < 0.05; ** P < 0.01, one-way ANOVA analysis. 

E–F, Data represent the means ± SEM of 5 mice. Data are representative of two independent experiments. * P < 0.05; ** P < 0.01, one-way ANOVA analysis.
Bezaﬁbrate and PD-1 Blockade Enhance CTL Survival and Proliferation

Bezaﬁbrate improves survival capacity of in vitro–stimulated CTLs

To analyze the mechanism by which PPAR signaling inhibits apoptosis, we investigated the effect of bezaﬁbrate on the survival capacity of CD8+ T cells in vitro. Naïve CD8+ T cells were stimulated as shown in Fig. 5A and expanded until day 13 in the presence of bezaﬁbrate or solvent (DMSO) control. The effect of bezaﬁbrate on T-cell longevity was tested in an over-activation-induced cell death system. After restimulation with anti-CD3 and anti-CD28 on day 13, bezaﬁbrate treatment signiﬁcantly reduced the number of apoptotic cells (Fig. 5B). Basal OCR and ECAR were not changed by bezaﬁbrate treatment on day 13, but SRC and the OCR/ECAR ratio were signiﬁcantly increased in the bezaﬁbrate-treated group, again indicating that bezaﬁbrate increased the survival capacity of CTLs (Fig. 5C). We conﬁrmed that bezaﬁbrate signiﬁcantly increased the expression of Bcl2, Birc3, and API5 genes involved in the apoptosis inhibition pathway (Fig. 5D). We further performed GeneChip analysis to identify or differentiate gene-expression signatures between solvent control and bezaﬁbrate-treated CD8+ T cells on day 13 (Supplementary Fig. S6A and S6B). KEGG pathway analysis demonstrated that bezaﬁbrate-treated cells on day 13 displayed differential changes among genes involved in various pathways such as PPAR signaling, fatty acid metabolism, AMPK signaling, cytokine–cytokine receptor interaction, chemokine signaling pathway, complement and coagulation cascades, natural killer cell–mediated toxicity, and metabolism (Supplementary Fig. S6C and S6D).

We focused on upregulated genes involved in preventing apoptosis and analyzed the protein interactome using the STRING database. We found that Bcl2 may be stabilized by interacting with Cpt1, which is also induced by PPAR signaling (ref. 28; Fig. 5E). PPAR regulates cyclic AMP response element binding (CREB), which enhances the expression of PGC-1α in a feedforward way (refs. 29–32; Fig. 5E). Indeed, both protein and mRNA of Cpt1α and CREB1 were upregulated in bezaﬁbrate-treated CD8+ T cells on day 13 (Fig. 5F and G).

Figure 3.
Bezaﬁbrate combination therapy enhances expression of genes associated with mitochondrial biogenesis and FAO in CD8+ T cells in vivo. A and B, MC38-bearing mice were treated with anti–PD-L1 and bezaﬁbrate on the same schedule as shown in Supplementary Fig. S1A. Mice were sacriﬁced on day 9, and CD8+ T cells isolated from DLN were pooled from 5 mice. PGC-1α, TFAM, Uqrc1, NDUSF8, ATP5a1, Cpt1b, LCAD, and MCAD expression was examined by quantitative PCR (qPCR) in DLN CD8+ T cells of treated groups. Data represent the means ± SEM of 3 wells assuming the untreated group = 1 in qPCR analysis. Expression in each group was compared with the anti–PD-L1 treated group. *, P < 0.05; **, P < 0.01; ***, P < 0.001, one-way ANOVA analysis.
Bezaflibrate increases the number of effector CTLs by enhancing their survival capacity and proliferation. A–D, CellTrace-labeled CD45.1⁺ CD8⁺ T cells were transferred into CD45.2⁺ CD8⁻ mice. The mice were inoculated with MC38 and treatment started 5 days after MC38 inoculation. Mice were sacrificed on day 9, and CD8⁺ CD45.1⁺ T cells in DLNs and tumor sites were analyzed. A, A schematic diagram of the experimental schedule. B, Representative FACS patterns stained with CD45.1 and CellTrace among the gate of CD8⁺ CD45.1⁺ T cells are shown (left). The frequencies of fully proliferated cells (tumor-reactive CTLs) were compared between groups (right). C, DLNs were stained with annexin V and propidium iodide (PI). Representative FACS profiles of annexin V and PI staining after gating on CellTrace⁺ CD45.1⁺ CD8⁺ T cells are shown (left and middle). Frequency of apoptotic cells (annexin V⁺ PI⁺) was compared between treated groups (right). D, CD8⁺ CD45.1⁺ T cells in TILs were intracellularly stained with Bcl2. Representative FACS profiles of Bcl2 staining are shown (left). Frequency and MFI of Bcl2⁺ T cells among CD8⁺ T cells are shown (right). E, MC38-bearing mice were treated with anti-PD-L1 and bezaflibrate on the same schedule as shown in Supplementary Fig. S1A. DLN cells on day 13 were stained with anti-CD8, anti-CD62L, anti-CD44, and Ki67. The number of Ki67⁺ T cells in CD8⁺ T cells is shown (left). Representative FACS profiles of P1–P3 among CD8⁺ T cells stained with Ki67 in mice treated with anti-PD-L1 and bezaflibrate (middle). The number of Ki67⁺ T cells in P1–P3 was compared between treated groups. Colors correspond to the P1–P3 populations (right). B–E, Data represent the means ± SEM of four or five mice. Data are representative of two independent experiments. *, P < 0.05; **, P < 0.01, one-way ANOVA analysis.
Bezaﬁbrate attenuates overactivation-induced apoptosis of CTLs in vitro. A, Naive CD8⁰ T cells (CD44⁻ CD8⁰ T cells) were isolated from the spleen of C57BL/6 mice and stimulated with anti-CD3 and anti-CD28 coated beads in presence of IL2 and bezafibrate on days 0 and 3. Cells were expanded in the presence of IL2 and bezafibrate until day 13 and used in the following experiments. B, T cells were restimulated on day 13 in the presence of bezafibrate for 24 hours. Annexin V and PI were used to stain live (annexin V⁻ PI⁻) and apoptotic cells (annexin V⁺ PI⁺). Representative FACS proﬁles of annexin V and PI staining are given (left). Frequency of live and apoptotic cells was compared between treated groups. C, OCR of day 13 cells was measured by the Seahorse XFe96 analyzer (left top). SRC were calculated (bottom left). OCR/ECAR ratio was measured (bottom right). D, Bcl2, Birc3, and API5 expression was examined by quantitative PCR (qPCR) on day 13. E, Estimated downstream factors of the PGC-1α/PPAR axis including Bcl2, Cpt1a, and CREB are shown based on the STRING database and literature. F–G, Cpt1a (F) and pCREB (G) expression was determined by western blotting (left) and qPCR (right) using T cells on day 13. Data represent the means ± SEM of 3 wells. "*, P < 0.05; "**, P < 0.01; "***, P < 0.001, two-tailed Student t test. C, Data represent the means ± SEM of 6 wells. "*, P < 0.05, two-tailed Student t test.
Bezaflurbirate promotes proliferation of in vitro–stimulated naïve CD8+ T cells

Our in vitro data suggest that enhanced proliferation of CTLs driven by the bezaflurbirate combination treatment contributes to increasing the number of effector killer T cells (Fig. 4E). To validate the effect of bezaflurbirate on T-cell proliferation, we stimulated naïve CD8+ T cells with anti-CD3 and anti-CD28 in the presence of bezaflurbirate or solvent (DMSO) control. As shown in Fig. 6A, in vitro–stimulated naïve CD8+ T cells in the presence of bezaflurbirate incorporated more 3H-thymidine than those without bezaflurbirate. The enhancement of proliferation by bezaflurbirate treatment was confirmed by the dye dilution experiment (Supplementary Fig. S7A). Because bezaflurbirate treatment improved mitochondrial activities in vivo, we also investigated whether enhanced CTL proliferation in vitro by bezaflurbirate is accompanied by mitochondrial activation. As shown in Fig. 6B, both OCR and ECAR were significantly increased, indicating that CTL reached a higher energy state with bezaflurbirate treatment. Although ATP turnover and glycolytic capacity were upregulated, SRC was decreased in the bezaflurbirate-treated group (Fig. 6C). As bezaflurbirate treatment enhanced OCR values, we investigated its effect on other mitochondrial activation parameters. We found that bezaflurbirate-treated cells possess larger mitochondrial areas, higher intensity of MitoTracker Deep Red, and more ROS than DMSO-treated cells, proving that mitochondria are activated during T-cell priming (Supplementary Fig. S7B). These data suggest that the effect of PPAR signaling on T-cell priming (day 2) is more associated with proliferation (anabolic pathway) than with longevity (catabolic pathway). Thus, PPAR signaling enhances proliferation during the early (priming) phase and inhibits apoptosis during the effector phase of the T cells.

Discussion

PD-1 blockade–based cancer immunotherapy has changed cancer treatment because it reaches more cancer targets and shows longer responses with fewer side effects than other cancer therapies (11, 33, 34). However, many patients do not respond to this therapy (12, 35). To enhance its efficacy and increase the range of patients who respond, combinatorial therapies involving PD-1 blockade have been designed and evaluated in clinical trials. One main cause of poor response is the absence or insufficiency of functional effector T cells at tumor sites, likely because of terminal differentiation and apoptosis mediated by PD-1 blockade (17, 27).

We found that bezaflurbirate treatment enhanced proliferation of CTLs and inhibited their apoptosis. Proliferation of primed CTLs generated more effector T cells. PPAR activation by bezafibrate enhanced both OCR and ECAR, explaining the upregulation of ATP production and glycolysis, both of which are necessary for induction of effector CTLs. These data are supported by studies showing that activation of both the mitochondria and PPAR/FAO pathways is necessary for proper activation of CTLs in the early stimulation phase (36, 37). The positive feed-forward loop in which PPAR signaling activates the mitochondria and enhances proliferation likely involves mtTOR activation and promotes cellular proliferation through protein synthesis (38). mtTOR signaling enhances cellular proliferation and AMPK activation improves cell survival (38). Therefore, the antiapoptotic and proproliferation effects of bezafibrate are indicated by our results showing that total CD8+ T cells from DLN isolated from mice treated with anti–PD-L1 and a mitochondria-activating chemical showed upregulation of both AMPK and mtTOR activity, even though mtTOR and AMPK compete with each other with in a single cell (20, 38).

Chronological dissection of the effects of PD-1 blockade and bezafibrate is schematically represented (Supplementary Fig. S8). Upon activation, naïve T cells proliferate and acquire effector function, a process that requires energy generation by glycolysis (Supplementary Fig. S8A). To avoid terminal differentiation and cell death induced by overactivation, T cells express immune checkpoints such as PD-1 and shift their energy metabolic pathway to FAO/OXPHOS (19, 27, 39). PD-1 signal confers T-cell longevity by compromising proliferation and killing activity, producing dysfunctional cells that may allow tumor cells to escape immune surveillance. In contrast, PD-1 blockade recovers T-cell effector function and reactivates effective antitumor immunity, which in turn promotes terminal differentiation and apoptosis accompanied by increased glycolysis and decreased FAO (Supplementary Fig. S8B; ref. 27). Thus, PD-1 blockade alone may reduce the number of functional effector T cells, which explain the incomplete responsiveness to PD-1 blockade. Introduction of pan-PPAR signaling in T cells by bezafibrate led to enhanced FAO, even in the PD-1 blocked state, and prolonged survival of CD8+ T cells (Supplementary Fig. S8C). PPAR activation also boosts the proliferation of CD8+ T cells, which increases the number of functional effector T cells (Supplementary Fig. S8C). Our transcriptome analysis showed that PPAR pathway activation rescues PD-1 blockade–induced apoptosis in T cells by upregulating the antiapoptotic proteins Bcl2, Birc3, and API5. Gene-expression data revealed that the FAO pathway is promoted during PPAR stimulation, through increased expression of Cpt1, an enzyme key to fatty acid metabolism in mitochondria. Cpt1 is stabilized by interaction with Bcl2, and inhibition of Cpt1 enhances cell death (28). Our results suggest that PPAR inhibits apoptosis of CTLs by upregulating Bcl2, Cpt1, and cross-talk between PPAR and Bcl2 (29). The effect of the PPAR pathway on T-cell longevity is indicated by the improvement in mitochondrial SRC defined as the potential ATP levels in OXPHOS that can be used when energy demand surges. Thus, SRC enhancement in CTLs by bezafibrate suggests that CTLs can survive longer and increase their number in a memory-like pool (23). We observed more depolarized mitochondria (~5.2%) in the TIL under anti–PD-L1 plus bezafibrate therapy (Fig. 2F). This mitochondrial population characterized by low Mitotracker Deep Red and high MitoTracker Green supports longevity of T cells because cells with depolarized mitochondria would undergo mitophagy to eliminate damaged mitochondria and enhance cellular survival (40–42). Thus, bezafibrate treatment, which depolarizes mitochondria, would extend the life span of cells, as we observed.

PPAR signaling induces expression of a range of target genes (43). Our microarray data from CD8+ T cells showed that many of the genes identified belong to energy metabolism-related pathways, e.g., Cpt1, LCAD, MCAD, hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), β-subunit (Haldib), malic enzyme 1 (Me1), Perilipin 2 (Plin2), and stearoyl-CoA desaturase-1 (Scd1). Our data also revealed additional genes induced by PPAR signaling. For instance, genes in pathways related to immune function were induced, including cytokine–cytokine receptor interactions, the...
chemokine signaling pathway, complement and coagulation cascades, and natural killer cell–mediated toxicity. Therefore, our data suggest additional mechanisms for regulating the killing activity of CD8\(^+\) T cells by PPARs.

We found that each subset of the T-cell population (P1/P2/P3) exhibited different mitochondrial activities. However, the P3 population likely has a large contribution to the changes observed in the flux assay of total CD8\(^+\) T cells for the following reasons. We demonstrated that, after bezafibrate treatment, mitochondrial activity was increased in the P3 population but not in others. Although naïve and memory populations (P1 and P2) are represented in T cells, mitochondrial activities do not change in these populations after the bezafibrate combination treatment. Further, the bezafibrate combination therapy increased the number of cells only in the P3 population. Therefore, it is likely that upregulation in the flux assay in the total CD8\(^+\) T cells was mainly due to metabolic changes of the P3 population.

We previously showed that metabolic genes in tumor cells were unperturbed by treatment with Luperox or uncoupler alone (20). We expected that bezafibrate would not have large effect on tumor metabolism, as the dose used in this study was less than one-tenth the dose showing cytotoxicity toward tumor cells (44). If bezafibrate exerted its antiapoptotic effect or proproliferation effect on tumor cells, tumor growth would be faster compared with the untreated group. However, we observed no enhanced tumor growth \textit{in vivo} when bezafibrate was administered alone, suggesting no direct effect of bezafibrate alone on tumor cells.

In summary, we showed that PPAR signaling reprograms CTL energy metabolism and overcomes the reduction in functional effector T-cell number associated with PD-1 blockade by decreasing apoptosis or increasing proliferation. Apoptosis was inhibited by upregulation of the antiapoptotic factor Bcl2 in CTLs. Enhanced proliferation can be explained by mitochondrial activation and upregulation of the mTOR pathway. Our
results will enable development of improved combination therapies and allow for the treatment of patients who are less responsive to therapy blocking the PD-1/PD-L1 interaction alone.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: P.S. Chowdhury, K. Chamoto, T. Honjo
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PPAR-Induced Fatty Acid Oxidation in T Cells Increases the Number of Tumor-Reactive CD8 + T Cells and Facilitates Anti-PD-1 Therapy

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