Ketogenic Diet and Ketone Bodies Enhance the Anticancer Effects of PD1 Blockade

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Abbreviations:

Brief Summary (max 25 words): Ketogenic diet induces a 3-hydroxybutyrate-mediated antineoplastic effect that relies on T-cell mediated cancer immunosurveillance, paving the way to accelerated responses to PD1-blockade.

Abstract

Limited experimental evidence bridges nutrition and cancer immunosurveillance. Here, we show that ketogenic diet (KD) or its principal ketone body, 3-hydroxybutyrate (3HB), most specifically in an intermittent scheduling, induced T cell-dependent tumor growth retardation of aggressive tumor models. In conditions in which anti-PD-1, alone or in combination with anti-CTLA-4, failed to reduce tumor growth in mice receiving a standard diet, KD or oral supplementation of 3HB reestablished therapeutic responses. Supplementation of KD with sucrose (which breaks ketogenesis, abolishing 3HB production) or with a pharmacological antagonist of the 3HB receptor GPR109A abolished the antitumor effects. Mechanistically, 3HB prevented the ICB-linked upregulation of PD-L1 on myeloid cells while favoring the expansion of CXCR3+ T cells. KD induced compositional changes of the gut microbiota with distinct species such as Eisenbergiella massiliensis commonly emerging in mice and humans subjected to carbohydrate low diet interventions and highly correlating with serum concentrations of 3HB. Altogether, these results demonstrate that KD induces a 3HB-mediated antineoplastic effect that relies on T-cell mediated cancer immunosurveillance.
Introduction

The clinical optimization of immune checkpoint inhibitors has provoked a paradigm shift in the treatment of advanced cancers, implementing immunotherapy as a first- and second-line modality across many tumor types. The major advance of immunotherapy concerns the long-term increase in overall survival, as observed for treatment with antibodies targeting CTLA-4 (cytotoxic T-lymphocyte-associated protein 4), PD-1 (Programmed cell death 1), or PD-L1 (Programmed death-ligand 1), compared to other standard therapies such as chemotherapy or tyrosine kinase inhibitors. However, two limitations affect this medical breakthrough. First, only a subset of cancers within each indication (melanoma, lung, kidney, bladder, head and neck tumors...) responds to immune checkpoint blockage (ICB), leaving the majority of patients without clinical benefit. Second, immune-related adverse events are frequent, largely unpredictable and often debilitating, limiting the clinical use of ICB. Several factors, including host genetics, the tumor mutational load, as well as environmental cues, may determine immune responses against cancer. While most efforts to understand cancer immune resistance largely focused on somatic alterations of cancer cells and their effects on the tumor microenvironment and lymphoid organs, relatively little information is available about environmental factors affecting the delicate balance between metabolism, microbiota, immunity and oncogenesis. Potentially relevant environmental factors encompass diet, lifestyle (such as stress, exercise and circadian rhythm) and past or present exposures to infectious agents and xenobiotics. Among these, the most easily actionable factor is diet. Food not only represents a source of macronutrients and micronutrients required for fueling metabolism, but also affects the homeostatic maintenance of intestinal barrier integrity, the composition of microbiota and the function of the immune system. To date, a plethora of dietary patterns and functional supplements (coined “nutraceuticals”) have been assessed for potential health benefits.
including cancer prevention. In 2010, the United Nations Educational, Scientific and Cultural Organization (UNESCO) recognized the Mediterranean diet as an “Intangible Cultural Heritage of Humanity.” Calorie restriction reduces the risk of chronic disease including cancer and increases life expectancy by promoting autophagy. Aside from caloric restriction, low protein consumption may restrain inflammation and improve anticancer immunosurveillance. Recently, several studies have suggested positive therapeutic effects of very-low-carbohydrate ketogenic diets (KD) on several diseases including cancer.

First described by Hans Krebs, ketosis is a metabolic state in which the body retrieves energy from the catabolism of ketone bodies (KB), as opposed to the normal state when glucose, and to a lesser degree, fatty acids and amino acids, influence are the main source of energy. KB include acetylacetate (H3C–CO–CH2–COO–), β-D-hydroxybutyrate (H3C–CHOH–CH2–COO–) and volatile acetone (H3C–CO–CH3). KD are high-fat, moderate-protein and low-carbohydrate (usually less than 40 g/day) diets, and induce a surge in KB, in particular 3-hydroxybutyrate (3HB). This increase in 3HB also occurs after fasting, favoring mitochondrial respiration rather than glycolysis for energy metabolism. So far, the rationale to use KD to combat cancer was based on a cell-autonomous hypothesis. One hallmark of all cancer cells is the abnormal metabolic phenotype first described by Otto Warburg, which is characterized by a bioenergetic shift from oxidative phosphorylation towards glycolysis. The Warburg phenomenon is linked to the overactivation of the insulin/IGF-1-dependent phosphatidylinositol 3-kinase (PI3K)/AKT/mechanistic target of the rapamycin (mTOR) system, not only due to mutations in the genes that code for pathway proteins, but also due to chronic hyperglycemia and hyperinsulinemia. Hence, a potential therapeutic strategy might involve KD, which reduces glucose availability to tumor cells, while providing KB as an alternative bioenergetic fuel to normal cells. On theoretical grounds, this should result in
selective starvation of tumor cells, which are unable to adapt to ketone metabolism as a result of their acquired metabolic inflexibility and genomic instability. This is emphasized by various tumors display reduced levels of two enzymes involved in KB catabolism (O-hydroxy-butyrate dehydrogenase and succinyl-CoA-acetoacetate-CoA transferase)\textsuperscript{25–28}.

Several diseases involving alterations in mitochondrial metabolism, such as epilepsy\textsuperscript{29}, diabetes mellitus type II, obesity, neurological disorders and cancer, may benefit from KD\textsuperscript{30–33}. However, most of the knowledge on the beneficial effects of KD on cancer relies on a cell-autonomous vision of malignancy, as well as on epidemiological studies rather than experimental animal models. We and others highlighted the intertwined relationship between the composition of the microbiota and antitumor immune responses in advanced cancer bearers treated with immunotherapy\textsuperscript{8,34}. Hence, bridging the gap linking dietary habits to clinical outcomes following immunotherapy remains an open challenge.

Here, we use preclinical tumor models to address the functional impact of KD and KB on the host immune system and tumor immunosurveillance, highlighting the immunomodulatory properties of KD and the most abundant KB, 3HB. We found both that KD and 3HB influence the balance between immunostimulatory and immunosuppressive pathways, thereby improving the antitumor effects of ICB.
Results

Ketogenic diet (KD) retards tumor growth in aggressive tumor models. We analyzed the antitumor effects of an ad libitum nutritional KD (called "KetoCal") based on a 4:1 fat: protein ratio compared to a vitamin and mineral-matched control diet (normal diet, ND) in C57BL/6 and BALB/c mice reared in specific pathogen-free (SPF) conditions. KD was initiated the very day of tumor inoculation and pursued for 12 consecutive days, and tumor progression was monitored twice a week. As compared to ND, KD reduced the growth of transgenic melanoma (RET) inoculated orthotopically into the skin (Figure 1A, B). Moreover, KD delayed the progression of a luciferase-expressing renal cancer (RENCa) implanted into the subcapsular area of kidneys from BALB/c mice, preventing tumor outgrowth in less than half cases while the other animals harbored smaller tumors (Figure 1C, D). These effects advantageously compared with regimens of short-term starvation or food supplements that we used in the past to improve anticancer immunosurveillance.12,35

So far, in accordance with the literature dealing with other cancer models (35–39), KD attenuated tumor progression in orthotopic models of aggressive neoplasia.

3-hydroxybutyrate (3HB) is the bioactive metabolite of KD. There were no significant differences in food consumption or weight gain between mice fed KD versus ND (Figure 2A). To identify the metabolic changes associated with ad libitum KD in tumor bearers, we performed a comprehensive metabolomic profiling of plasma, liver and heart tissues from 24 RET melanoma-bearing mice receiving either ND or KD at day 10, time corresponding to disease control for most animals. Non-supervised hierarchical clustering and principal coordinate analyses (PCA) revealed major differences between ND and KD (Figure 2B-C,
Table 1). KB including acetoacetate, 2-hydroxybutyrate (2HB) and 3HB, as well as carnitine derivatives (such as 3-methylglutaryl carnitine and the acetyl carnitine/carnitine ratio), short-chain saturated carboxylic acids (such as behenic acid), and polyamines (in particular, N1-acetyl spermidine and the putrescine/ornithine ratio) were markedly elevated in the plasma and other tissues from mice fed with KD (Figure 2D, E, Figure S1, Table 1). In contrast, acetylcholine, a range of acyl phosphatidylcholines (PCae), palmitate (the most abundant saturated fatty acid), several mono-unsaturated fatty acids, propionate, and hippurate were significantly decreased by KD compared to ND (Figure 2B, Table 1) suggesting the catabolism of fatty acids through β-oxidation.

Importantly, the plasma levels of 3HB, but not those of acetoacetate, negatively correlated with the size of RET melanomas (Figure 2F). Driven by this correlation, we attempted to establish a cause-effect relationship between systemic KB and KD-mediated anticancer effects. For this, we replaced KD by administration of 3HB per os (3HBpo). While an established protocol (4.2% 3HB drinking water) caused a modest increase in plasma 3HB (0.5 and 1 mM), a higher dose (12.6 % 3HB) allowed to achieve KD-related serum levels ≥3 mM (Figure S2). Of note, in healthy individuals, blood concentrations of total ketones are generally less than 0.5 mmol/L (equivalent to 52.05 mg/L of 3HB), whereas after 6-7 days fasting blood ketone concentrations can be 5–7 mmol/L (equivalent to 520.5–728.7 mg/L of 3HB) called starvation ketosis. Blood ketone concentrations in diabetic patients with ketoacidosis may exceed 25 mmol/L. At this dose of 12.6 %, 3HBpo retarded RET melanoma growth as much as did KD, both in terms of kinetics and complete responses (Figure 3A-B). Similarly, when 3HB was injected systemically (intraperitoneally, ip) to obtain KD-like pharmacokinetics, it mimicked the antitumor effects of KD (Figure 3A and B, Figure S2). Of note, blockade of the 3HB receptor, GTP coupled receptor 109A (GPR109A) by means of mepenzolate bromide (C21H26BrNO3)
abolished the control of tumor progression by 3HB_{po} as well as by KD (Figure 3C). Similarly, when KetoCal, the usual KD, was supplemented with sucrose (5% in the drinking water), the induction of KB (3HB and acetate) was prevented and the anticancer effect of KD was lost (Figure 3D-F, Figure S3).

Altogether, we conclude that KD reduces the growth kinetics of aggressive orthotopic cancers as a standalone treatment modality, at least in part through the pharmacological activity of 3HB on its receptor GPR109A.

**Contribution of microbial shifts and T lymphocytes to KD-mediated tumor growth control.** Oral intake of food or supplements can be expected to influence the intestinal microbiota. Indeed, the 16S rRNA sequencing of stools harvested at day 10 of KD in RET tumor bearers revealed significant deviations in the beta diversity of operational taxonomic units (OTUs). Multidimensional Principal Coordinates Analysis (PCoA) based on the Bray Curtis Dissimilarity Index unraveled significant (p=0.001, PERMANOVA test) compositional differences in ND versus KD samples (Figure 4A). Bacterial taxa with differential abundance between ND and KD groups were used as input for the linear discriminant analysis (LDA) to calculate an effect size (LEfSe analysis). KD induced an overrepresentation of *Akkermansia muciniphila, Ruthenibacterium lactatiformans, Pseudoflavonifractor capillosus*, but a relative loss of more than 10 species belonging to the Lactobacillaceae family (Figure 4B and C, Figure S4A, B). When analyzing taxa of low prevalence that were not considered in the LEfSe analysis, we found that the relative abundance of *Eisenbergiella massiliensis* (Figure 4C, right panel) and *Turicibacter sanguinis* (Figure S4B) were increased and decreased, respectively, in the stools of KD-fed mice, compared with ND.
We next assessed the effects of broad-spectrum antibiotics (ATB) on the anticancer activity of KD and 3HB. ATB tended - albeit not significantly - to reduce the protective effect of both nutritional interventions against RET melanoma outgrowth (Figure 4D). In a Spearman correlation matrix integrating all bacterial taxa and KB, we found that *Eisenbergiella massiliensis* was at the center of the KB-related metacluster (Figure 5A). Indeed, its relative abundance in feces correlated with the plasma concentrations of the two KB (acetoacetate and 3HB), only in KD-treated mice, not in ND controls (Figure 5A-C, Figure S4C).

To analyze the clinical relevance of these findings, we turned to an epidemiological study linking food ingredients, systemic inflammatory markers and gut microbiota, the Personalized Responses to Dietary Composition Trial (PREDICT-1) that enrolled 1,000 participants (mono and dizygotic healthy twins from the Twins UK cohort, and non-twin healthy individuals). This trial consisted in collecting food questionnaires, plasma metabolomics and shotgun metagenomics data. We analyzed correlations between plasma KB concentrations, carbohydrate consumption (using an estimate of carbohydrates normalized to energetic contribution, carb%E) and the intestinal OTUs identified in the murine KD fingerprint. Again, *E. massiliensis* positively correlated with 3HB and acetoacetate (p<0.05) in humans, while *Bifidobacterium adolescens* and *Prevotella copri* anticorrelated with plasma KB levels (Figure 5D). *Turicibacter sanguinis* and *Pseudoflavonifractor capillosus* positively and negatively correlated with carb%E, respectively (Figure 5D).

Given the reported immunostimulatory capacity of distinct OTUs selected from the KD (such as *A. muciniphila*) 42, we next analyzed the effects of T cell depletion on the tumor growth-reducing effect of KD and 3HB. Both nutritional interventions lost their anticancer effects in mice injected with antibodies against CD4 and CD8 (Figure 6A).
In conclusion, KD and 3HB mediate their antitumor activity not through direct, cancer cell-autonomous mechanisms but rather through effects on the host immune system.

**Synergistic antitumor effects of ketogenic interventions with immune checkpoint blockade.** Driven by the consideration that KD or 3HB induce T cell-dependent anticancer effects, we combined these nutritional interventions with the most powerful combination of immunostimulatory monoclonal antibodies (mAbs) in the clinics, thus targeting both PD-1 and CTLA-4. In the RET melanoma model, diet-based host conditioning increased the efficacy of combination ICB (cICB: anti-PD-1 + anti-CTLA-4), with significant differences observed as early as after the first systemic injection of cICB (Figure 6B, C). In the context of complete cICB regimen (3 ip injections over a 6-day period), 3HB_{po} boosted the tumor growth-inhibitory activity of cICB, prolonging overall survival of almost 60% RET tumor bearers (Figure 6D). 3HB_{po} was superior to KD, although both nutritional interventions led to an increase in the cICB-induced pool of Tc1 (CXCR3^{+}CD8^{+}) splenocytes (Figure 6E). Of note, both diet interventions tended to mobilize the recirculation of patrolling activated monocytes by day 5 (Figure S5C and S5D). The improvement of cICB-induced tumor growth reduction conferred by KD, was lost upon supplementation with sucrose, which breaks ketogenesis (Figure 6F).

Given that most nutritional interventions are prescribed in intermittent courses to limit the constraints on the users, we compared continuous versus discontinuous (one week on, one week off) regimens of the KD or 3HB_{po} (Figure 7A, B) in the RET tumor model. Surprisingly, the combined effect of 3HB_{po} and cICB tended to be favored by the intermittent schedule (Figure 7B). In this latter setting, flow cytometry analyses of splenocytes revealed that the
On/off regimen of 3HBpo alone decreased the expression of activation/exhaustion markers (such as CTLA-4 and CD223, best known as Lag-3) on CD4+ T cells (Figure S5A) and prevented the cICB-induced up-regulation of Lag-3 and CD137 (best known as 4-1BB) on CD8+ T cells (Figure S5B). In contrast, the combination of cICB and intermittent 3HBpo boosted the expression of CTLA-4 on CD8+ T cells but simultaneously reduced the expression of CD86, the CTLA-4 ligand, on splenic myeloid cells (Figure 7C, D). Conversely, while PD-1 remained stable, the expression of its ligand PD-L1 was significantly decreased on splenic macrophages during the combinatorial therapeutic regimen (Figure 7E). To confirm that 3HBpo contributes to restrain PD-L1 expression despite the presence of a pool of Tc11 cells producing the PD-L1 promoting cytokine interferon gamma (IFNγ) in vivo 43, we exposed bone marrow derived-myeloid antigen-presenting cells to 3HB prior to exposure to rIFNγ in vitro. Indeed, low (micromolar) concentrations of 3HB reduced the IFNγ-induced upregulation of PD-L1 membrane expression but not that of major histocompatibility complex class II molecules (MHC II) (Figure 7F, G). Of note, 3HB did not modulate IFNγ-induced upregulation of PD-L1 membrane expression on tumor cells (Figure S5C). In summary, when combined with ICB, ketogenic regimens (KD or 3HB) induced the splenic accumulation of CXCR3+ Tc1 cells, as well as the upregulation of PD-1 and CTLA-4 on CD8+ T cells, but prevented the expression of their ligands on splenic macrophages, setting the stage for prolonged systemic T cell activation.

We next turned to orthotopic models of established carcinomas to evaluate the efficacy of KD in the context of ICB. Established RENCA renal cancers were best controlled when their BALB/c hosts were treated with cICB plus KD or 3HB, but only when the nutritional interventions were applied intermittently (Figure 8A, Figure S6A). We next selected an aggressive orthotopic TC-1 lung cancer tumor model, which is primarily resistant to anti-PD-1 or anti-CTLA4 Abs, as standalone therapies (Figure S6B). Importantly, the only efficient
regimen that lead to the eradication of established tumors (in >70% of the cases), allowing for long-term survival of most of the C57BL/6 hosts, was the combination of intermittent 3HBpo and anti-PD-1 mAbs (Figure 8B-C-D). Mice that had been diagnosed with TC-1 lung cancers (by bioluminescence imaging) and then cured by the combination of 3HBpo and anti-PD-1 resisted subcutaneous (sc) re-challenge with TC-1 cancers, yet readily developed MCA205 fibrosarcomas, indicating that they developed a specific long-term protective immune response (Figure 8E-F, Figure S6C).

Altogether, these results support the idea that ketogenic regimens can enhance the anticancer effects of ICB in multiple orthotopic tumor models including melanoma, renal and non-small cell lung cancer.
**Discussion**

So far, the rationale to use KD for combating neoplasia was based on a cell-autonomous hypothesis. Here, we highlight a non-cell autonomous mechanism of action of KD that can be exploited to ameliorate anticancer therapies. KD and more specifically 3HB induce immunostimulatory effects in secondary lymphoid organs that are important for natural and therapy-induced immunosurveillance.

Oral administration of 3HB increased the expansion of CD8+ T cells elicited by ICB while restraining the expression of activation/exhaustion markers such as CTLA-4, Lag-3, and 4-1BB/CD137 in the spleen. Concomitantly, 3HB limited the IFNγ-induced PD-L1 and CD86 expression on splenic macrophages in vivo (for both) and in vitro (for PD-L1), then reducing a negative feedback signal operating after T cell receptor (TCR) engagement. The poor expression of PD-1 and CTLA-4 receptor ligands may maintain T cell activation and fitness in secondary lymphoid organs. Of note, pioneering work highlighted the pivotal role of PDL-1 expression by antigen presenting cells in the efficacy of PD-1 blockade suggesting that KD may be pivotal to prime the host for a full blown immune response to immune checkpoint inhibition. One single report indicated the capacity of KD to elicit systemic innate immune responses through IL-17 producing γδT cells during virus infection. However, in this report, KB including 3HB could not substitute for KD to stimulate antiviral immunity. This is different from our observation that KD and 3HB have similar immune-dependent antitumor effects. Moreover, in tumor-bearing mice, we failed to observe any effects of KD on γδT and Natural Killer (NK) innate effectors (not shown).
Importantly, KD and 3HB slowed natural tumor progression in the absence of additional therapeutic intervention, but also accelerated and improved the efficacy of cICB against established and aggressive orthotopic melanoma, lung and renal cell cancers. This anticancer effect was blunted by sucrose supplementation (in the case of KD) or by blocking the 3HB receptor (for both KD and 3HB). Indeed, T cells and GPR109A engagement were mandatory for the full-blown anticancer properties of 3HB, paving the way to the accelerated efficacy of ICB.

As expected, KD markedly affected the composition of the gut microbiota, shifting the balance from tolerogenic (Lactobacilli spp., C. asparagiforme) towards immunogenic bacteria (such as A. muciniphila)\textsuperscript{48}. Of note, immunogenic bacteria can share antigens with oncogenic drivers and boost the cognate T cell arm of immune responses\textsuperscript{49}. However, several arguments indicate that the modulation of the microbiota does not play a central role in the anticancer effects of KD. First, antibiotics did not significantly compromise the anticancer efficacy of the KD or 3HB. Second, the intraperitoneal (ip) that mimics parenteral administration in mice of 3HB recapitulated the findings obtained with KD. Third, we failed to observe additive effects of prebiotics and probiotics in the same cancer models (not shown). All these lines of evidence argue in favor of a direct (microbiota-independent) immunostimulatory action of KD.

Interestingly, our data appear to indicate that intermittent administration of 3HB might be more beneficial than the continuous regimen, alone or in combination with cICB. A large body of literature reports that intermittent fasting, which leads to discontinuous ketogenesis, is associated with improved health\textsuperscript{50–52}. Moreover, intermittent KD can improve the health span of mice more efficiently than continuous KD\textsuperscript{53}. Indeed, cyclic KD may prevent high fat diet-associated obesity, resulting in long term maintenance of normal weight and cyclic KD resulted
in higher plasma levels of betahydroxybutyrate than continuous KD, particularly during daytime \textsuperscript{53}. More work will be needed to delineate the immunological and/or metabolic mechanisms underpinning these effects in cancer patients.

Regardless of these limitations, the present work has important implications, as previously discussed, not only for obese individuals and athletes, but also in brain and endometrial cancer patients \textsuperscript{54-56}. KD might be implemented prior, or concomitantly, to anticancer therapies (including immunogenic chemotherapy and immunotherapy) with the scope of potentiating their immunostimulatory effects, reducing the number of therapeutic cycles and achieving a higher rate of complete cures. Given the poor palatability of KD and the consequent lack of compliance, as well as the potential toxicity of KD on the cardiovascular system \textsuperscript{57}, substitution of KD by its main metabolite 3HB represents an attractive option. Numerous clinical trials are underway to evaluate KD either as a standalone cancer treatment for instance in children with brain tumors (NCT03328858, NCT03955068) or adults with advanced cancers (NCT01716468), or in association with other treatments such as chemotherapy (NCT03535701) and hormonotherapy (NCT03962647) in breast cancers, as well as chemoradiation in lung cancers (NCT01419587). Only one trial is investigating the effect of a ketogenic drink on perioperative and oncologic outcomes in cancer patients (NCT03510429).

Prospective randomization, and appropriate pharmacodynamics markers will be necessary to adjust dosing and scheduling as well as the clinical endpoints of these studies. Our findings emphasize that not only metabolic and inflammation-related parameters but also immunological parameters must be evaluated to appreciate the impact of nutritional interventions on cancer.
Methods

Mice. Female C57BL/6JOlaHsd and BALB/cJRj were purchased from Harlan (Gannat, France) and Janvier (Le Genest-Saint-Isle, France), respectively. Mice were used between 8 and 16 weeks of age. All mice experiments were performed at Gustave Roussy Cancer Campus and mice were housed in specific pathogen-free conditions or maintained in isolators.

Cell lines. RET melanoma (a transgene-enforced expression of the Ret protooncogene under the control of the metallothionein-1 promoter driving spontaneous melanogenesis, kindly provided by Professor Viktor Umansky, from DKFZ - German Cancer Research Center (Germany)) (syngeneic from C57BL/6J mice), luciferase expressing TC1 cell line (TC-1_luc) was kindly shared by Dr. Tzyy-Choou (The Johns Hopkins Hospital (Baltimore, USA). from Wu and luciferase-transfected renal cancer (RENCA) cell lines (syngeneic for BALB/c mice, kindly provided by Transgene, Illkirch, France) were cultured at 37°C under 5% CO₂ in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin, 2 mM L-glutamine and 1% of sodium pyruvate and non-essential amino acids (all from Gibco-Invitrogen), referred herein as complete RPMI medium. Cell lines were regularly tested for mycoplasma contamination and cells were not used for more than 10 passages.

Diet. Mice were randomized according to their weight before beginning of the diet and assigned to control [Normal Diet (ND)] or Ketogenic Diet (KD) or 3-ß-hydroxybutyric acid (3HB) per os (3HBpo) or intraperitoneally (ip) 3HB (3HBip) groups. C57BL/6 mice started their diet the very day of subcutaneous inoculation with RET. BALB/c mice were fed different diets, ND, KD, 3HBpo ten days before the RENCA inoculation into the socalpsular area of the kidney. The ketogenic diet (KD) is apostrophed KetoCal® by the vendor Nutricia (Erlangen, Germany) to whom we purchased the product. KetoCal® is a nutritionally complete ketogenic formula with a ketogenic ratio (fats: proteins + carbohydrates) of 4:1. The fat was derived from soybean-oil.
KetoCal® diet was fed to mice in a paste form (water: KetoCal®; 1:2) within the cage. We freshly prepared the mixture corresponded to 10g of KD per day. The diets were changed three times a week. Mice in the "3HB po groups" received ND in food but drinking water replaced by ad libitum Ketoforce purchased from Ergomax (Nijmegen, Netherlands). We freshly prepared the mixture corresponded to 10g of KD per day. The diets were changed three times a week. Mice in the "3HB po groups" received ND in food but drinking water replaced by ad libitum Ketoforce (purchased from Ergomax), which was mineral salts 3HB solution at a final concentration of 4.2 mL in 100 mL of water. It corresponds to 16.38 g of 3HB in 100 mL of water. 3HB po solution was changed three times a week to maintain the stability of the product during the entire experiment. Mice in "3HB ip" groups received ND in food and regular drinking water but were injected ip with β-Hydroxybutyric acid sodium salt purchased at Sigma Aldrich (St. Louis, MO, USA). We prepared extemporaneously the 3HB ip at 178 mg/mL in PBS 1X. The injections were performed daily during 8 days every other week (the "On / off" protocol).

For animals inoculated with TC-1-luc cells, treatment of 3HB (3x=12% KetoForce in drinking water) was initiated 9 days before tumor inoculation, in an On-off dotted mode (3-day 3-HB solution/3-day water). After TC-1 injection, the treatment of 3HB was maintained in the On-off system during the whole experiment. For the experiments aimed at interrupting ketosis, sucrose (C12H22O11, Sigma Aldrich) was added in the water at a final concentration of 5% in drinking water. The bottle was changed three times a week to avoid contamination.

**Tumor models. Subcutaneous RET melanoma.** C57BL/6 mice were subcutaneously (sc.) injected in their flank with 0.5 x 10⁶ RET cells. Tumor size was routinely monitored every 3 days using a caliper. When tumors reached 28 to 30 mm² in size, tumor-bearing mice were randomized to receive a cICB i.e neutralizing mAbs anti-PD-1 (clone RMP1-14, 250 µg/mouse), and anti-CTLA-4 (9D9, 100 µg/mouse). Mice were injected ip with cICBs or
combined isotype control antibodies (clone 2A3 and MPC11) 3 times at 3-day intervals. All mAbs and the recommended isotype control mAbs for in vivo use were obtained from BioXcell (West Lebanon, NH, USA).

**Renca-luc orthotopic kidney cancer model.** The day of tumor challenge, mice were anesthetized with isofluorane, then $10^4$ RENCA tumor cells resuspended into 30 µL of PBS were injected into the subcapsular space of the right kidney. The skin incision was then closed with surgical clips. Tumor incidence (at day 7) and development were monitored by in vivo photonic imaging of tumor cell luciferase activity. Briefly, mice received an ip. injection of the substrate of luciferase (E1605, Promega, Walldorf, Germany) at a dose of 3 mg/kg and in vivo photonic imaging was acquired with an IVIS Spectrum Imaging Series (Perkin Elmer, Whaltham, USA). Tumor-bearing mice were randomized to designed groups for treatments with PD-1 or CTLA-4 blocking antibodies, or equivalent isotype control antibodies at day 7 after tumor challenge (as previously). Tumor growth was monitored once weekly.

**TC-1_luc orthotopic lung cancer model.** To establish the orthotopic lung cancer model at day 0, TC-1_luc cells ($5 \times 10^5$ /mouse, in 100 µL PBS) were intravenously injected to wild type C57BL/6 mice (8 weeks old female). Tumor incidence was monitoring at day 10 and mice were randomized between ICB or cICB treated groups or isotype control Ab groups. Photonic imaging was acquired on a Xenogen IVIS 50 bioluminescence imaging system for TC1 model. Mice were injected ip with anti-PD-1 (29F.1A12, 200 µg/mouse) or with anti-CTLA-4 (9D9, 100 µg/mouse) or cICB or isotypes controls. All mAbs and the recommended isotype control mAbs for in vivo use were obtained from BioXcell (West Lebanon, NH, USA). The ICB treatment was administrated 4 times with 4-day intervals. In vivo imaging was acquired every 3–5 days with the exposure time 4 min, then 2 min and 1 min if saturation occurred. 3HB dosing was 3x the dosing utilized for RET, given the pharmacokinetics depicted in Figure S2C.
**T cell depletion.** T cell depletion was performed by ip treatment with depleting anti-CD4 and anti-CD8 mAbs (GK1.5 and 53–6.72; 200μg/mouse) or respective isotype controls (LTF-2 and 2A3). All mAbs and the recommended isotype control mAbs for in vivo use were obtained from BioXcell (West Lebanon, NH, USA). Depletion treatment started 4 days before RET challenge and repeated at the same dose every 7 days. In each group, tumor growth was monitored three times per week.

**Antibiotic treatments.** Mice were treated with an antibiotic solution (ATB) containing ampicillin (1 mg/ml), streptomycin (5 mg/ml), and colistin (1 mg/ml), with or without the addition of vancomycin (0.25 mg/ml) via the drinking water (all purchased in Sigma-Aldrich). Solutions and bottles were replaced 3 times and once weekly, respectively. In brief, mice were treated with ATB throughout the experiment, with the addition of vancomycin for the first 4 days with ATB and without the addition of vancomycin continuously throughout the experiment from then on. Antibiotic activity was confirmed by cultivating fecal pellets resuspended in PBS 1X at 0.1 g/mL on COS plates (5% Sheep Blood Columbia Agar) plates for 48 h at 37°C in aerobic and anaerobic conditions.

**Feces and plasma collection.** Plasma and feces were harvested in each mouse and group for metagenomics and metabolomics, respectively, at day 10, day of RET inoculation, or after completion of immunotherapy. Blood was drawn on EDTA after mouse anesthesia in the submandibular area, then centrifuged 10 min at 5000 G to allow plasma harvesting. Samples were stored at -80°C until processing.

**Metabolomics sample preparation.** About 30 mg of tissue for each condition were first weighted and solubilized into 1.5 mL microtubes with ceramic beads with 1 mL of cold lysate
buffer with ISTD (MeOH/Water/Chloroform, 9/1/1, -20°C), homogenized, and centrifuged. Then the upper phase of the supernatant was split in three parts: GC-MS experiment, short chain fatty acids (SCFA) and ketone bodies experiment, and others reversed phase UHPLC-MS experiments. Rest of the supernatant was spiked with methanol (2% of sulfosalicylic acid), centrifuged, evaporated and recovered with MilliQ water before injection in UHPLC/MS of the polyamines method. GC-MS aliquot was evaporated and metabolites were derivatized with methoxyamine in pyridine and N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA). SCFA and ketone bodies were derivatized with 3 nitrophenylhydrazine (3-NPH) and N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC). Reversed phase LC-MS aliquots were evaporated and solubilized with MilliQ water. Regarding plasma preparation, a volume of 25 µL were mixed with 250 µL same cold solvent mixture as above, into 1.5 mL microtube, and prepared in a similar way than tissues. GC-MS/MS method was performed on a 7890B gas chromatography (Agilent Technologies, Waldbronn, Germany) coupled to a triple quadrupole 7000C (Agilent Technologies, Waldbronn, Germany) equipped with a High sensitivity electronic impact source (EI) operating in positive mode. Helium gas flowed through column (J&WScientificHP-5MS, 30m x 0.25 mm, i.d. 0.25 mm, d.f., Agilent Technologies Inc.) at 1 mL/min. Targeted analyses of bile acids and polyamines were performed separately on a RRLC 1260 system (Agilent Technologies, Waldbronn, Germany) coupled to a Triple Quadrupole 6410 (Agilent Technologies) equipped with an electrospray source. Collision gas was nitrogen. Scan mode used was the multiple reaction monitoring (MRM). Peak detection and integration of the analytes were performed using the Agilent Mass Hunter quantitative software (B.07.01). Targeted analysis of SCFA and ketone bodies were performed on a RRLC 1260 system (Agilent Technologies, Waldbronn, Germany) coupled to a 6500+ QTRAP (Sciex, Darmstadt, Germany) equipped with an electrospray ion source. The instrument was operated using MRM. The software used to operate the mass spectrometer was Analyst (Version 1.7).
Peak detection, integration and quantification of the analytes were performed using MultiQuant quantitative software (Version 3.0.3). The profiling experiment was performed with a Dionex Ultimate 3000 UHPLC system (Thermo Scientific) coupled to a Q-Exactive (Thermo Scientific) equipped with an electrospray source operating in both positive and negative mode and full scan mode from 100 to 1200 m/z. The mass spectrometer was calibrated with sodium acetate solution dedicated to low mass calibration. Peak detection and integration were performed using the Thermo Xcalibur quantitative software (Version 2.2).

**Calibration curve preparation of 3-hydroxybutyric acid in plasma (absolute quantification).** Since basal concentrations of 3-HB can be monitored in the plasma of animals prior to any diet intervention, metered addition method was used for absolute quantification analysis. To prepare calibration samples, stock solution of 3HB was diluted in 25 µl of mice plasma (lithium heparin) to obtain 6 calibration points with concentrations of 46.49, 93.36, 237.06, 464.92, 933.57, 9335.7 µM, respectively. Calibration curve was prepared the same day and treated the same way than the SFCA and ketone bodies method sample aliquots: after protein precipitation, 40 µl of supernatant were derivatized and injected during the same batch of sample analysis. Measurement of 3HB was performed as previously described, on the 1260 system (Agilent) coupled to a 6500+ QTRAP (Sciex). MRM transition of the 3HB was Q1 m/z 238 to Q3 m/z 137. Absolute concentrations (in µM) of 3-HB were calculated from ion signal areas, with the calibration curve using a linear regression type. Accepted accuracies of calibration points were fixed between 80 and 120%, excepted for the lowest limit of quantification (LLOQ), which was set between 85 and 115%. In addition, calibration curve was split into two ranges: low (from 46.49 µM to 464.92 µM) and high concentration (from 237.06 µM to 9335.7 µM). Each calibration curve (low and high) should have a least 4 points, respectively. Their ranges should cover calculated concentrations of biological samples, except
basal ones. In this later case, concentrations were obtained with ratio related to the lowest calibration point.

**Fecal DNA extraction and microbiota characterization.** Preparation and sequencing of fecal samples were performed at IHU Méditerranée Infection, Marseille, France. Briefly, DNA was extracted using two protocols. The first protocol consisted of physical and chemical lysis, using glass powder and proteinase K respectively, then processing using the Macherey-Nagel DNA Tissue extraction kit (Duren, Germany) \(^6^0\). The second protocol was identical to the first protocol, with the addition of glycoprotein lysis and deglycosylation steps \(^6^1\). The resulting DNA was sequenced, targeting the V3–V4 regions of the 16S rRNA gene as previously described \(^6^2\). Raw FASTQ files were analyzed with Mothur pipeline v.1.39.5 for quality check and filtering (sequencing errors, chimeras) on a Workstation DELL T7910 (Round Rock, Texas, United States). Raw reads (16733247 in total, on average 120383 per sample) were filtered (6908784 in total, on average 49703 per sample) and clustered into OTUs, followed by elimination of low-populated OTUs (till 10 reads) and by de novo OTU picking at 97% pairwise identity using standardized parameters and SILVA rDNA Database v.1.19 for alignment. In all, 524 bacterial species were identified. Sample coverage was computed with Mothur and resulted to be on average higher than 99% for all samples, thus meaning a suitable normalization procedure for subsequent analyses. Bioinformatic and statistical analyses on recognized OTUs were performed with Python v.2.7.11. The most representative and abundant read within each OTU (as evidenced in the previous step with Mothur v.1.39.5) underwent a nucleotide Blast using the National Center for Biotechnology Information (NCBI) Blast software (ncbi-blast-2.3.0) and the latest NCBI 16S Microbial Database accessed (ftp://ftp.ncbi.nlm.nih.gov/blast/db/). A matrix of bacterial relative abundances was built at
each taxon level (phylum, class, order, family, genus, species) for subsequent multivariate statistical analyses.

**Microbiota and OTU-level analyses.** Measurements of α diversity (within-sample diversity) such as observed_otus and Shannon index, were calculated at OTU level using the SciKit-learn package v.0.4.1. Exploratory analysis of β-diversity (between sample diversity) was calculated using the Bray-Curtis measure of dissimilarity calculated with Mothur and represented in PCoA, while for Hierarchical Clustering Analysis (HCA) ‘Bray-Curtis’ metrics and ‘complete linkage’ method were implemented using custom scripts (Python v.2.7.11). In order to compare the microbiota taxa with gene expression datasets, a multivariate statistical Spearman correlation analysis (and related P values) was performed with custom Python scripts. Mann-Whitney U and Kruskall-Wallis tests were employed to assess significance for pair-wise or multiple comparisons, respectively, taking into account a P≤0.05 as significant. Correlations among relative abundance of candidate species and selected metabolites were estimated using Pearson correlation coefficient after a two-stages Benjamini-Hochberg false detection rate (FDR) of 10%. ANalysis Of SIMilarity (ANOSIM, which represents the difference of datasets’ centroids) or, when indicated, Pearson correlation coefficient, were computed with Python 2.7.11. LEfSe analysis was used to compare abundances of all bacterial species according to diet using the Kruskal-Wallis test (statistical significance was defined as P≤0.05 after a two-stages Benjamini-Hochberg false detection rate (FDR) of 10%). Bacterial taxa with differential abundance between study groups were used as input for the linear discriminant analysis (LDA) to calculate the effect size. LEfSe analysis at species level was performed with Mothur v.1.39.5 and graphed with Python 2.7.11.

**Flow cytometry on spleen and blood.** Spleens were harvested at the end of the experiment at early (day 12) or late (d17) timepoint. Spleens were crushed in RPMI medium and subsequently
filtered through a 100 μm cell strainer. In all cases, two million cells were pre-incubated with purified anti-mouse CD16/CD32 (clone: 93, BioLegend, REF# 16-0161-86) for 20 minutes at 4°C, before membrane staining. For intracellular staining, four million cells were used. Dead cells were excluded using the Zombie Aqua™ Fixable Viability Kit (BioLegend, REF# 423102) and the eBioscience™ Foxp3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent (ThermoFischer REF# 00-5521-00) was used. Anti-mouse antibodies (clones, company, references) used for phenotyping T cells (TH1, Tc1, Treg) were: CD45 (30-F11, BioLegend, REF# 103125), CD3e (145-2C11, BD Biosciences REF# 560527), CD4 (GK1.5, BioLegend, REF# 100421), CD8a (53-6.7, BioLegend, REF# 100761), CXCR3 (FAB1685P, ThermoFischer REF#17-1831-82), CD25 (PC61, BioLegend, REF# 102038), CTLA4 (UC10-4B9, ThermoFischer REF#12-1522-83), FOXP3 (FJK-16s, ThermoFischer REF#56-5773-82). For phenotyping T cell exhaustion, the anti-mouse antibodies were CD45 (30F11, BioLegend, REF# 109822), CD3 (17A2, BioLegend, REF# 100222), CD4 (GK1.5, BioLegend, REF#100428), CD8a (53-6.7, BioLegend, REF# 100722), Tim3 (B8.2C12, BioLegend, REF# 134014), Lag 3 (C9B7W, BioLegend, REF#125210), CD137 (4-1 BB) (17B5, ThermoFischer REF# 12-1371-83), CD279 (PD1) (J43, ThermoFischer REF# 11-9985-85). For phenotyping dendritic cells, the anti-mouse antibodies were CD45 (30F11, BioLegend, REF# 109822), CD3 (145-2C11, BioLegend, REF#100326), CD4 (GK1.5, BioLegend, REF#100428), CD8a (53-6.7, BioLegend, REF# 100761), CD11c (N418, BioLegend, REF# 117318), F4/80 (BM8, BioLegend, REF# 123117), CD11b (M1/70, BioLegend, REF#101257), PD-L1(CD274) (MIH5, ThermoFischer REF# 12-5982-82) , CD86 (GL1, BD Biosciences REF# 553691). Blood are harvested from mice 5 days after tumor and diet challenging. The blood is lysed by ACK and blood are stained with CD45 (30-F11, Biolegend, REF# 103128 ) , and negative selection with CD3 (17A2, Biolegend, REF# 100222), CD19 (6D5, Biolegend, REF# 115530) and NK (PK136, eBiosciences, REF# 108724). We selected monocytes with
CD11b FITC (M1/70, ThermoFischer, REF#11-0112-41), CD11c (HL3, BD, REF# 550283) and we observed the activation with CD69 (H1.2F3, BD Bioscience, REF#551113), CX3CR1 (SA011F11, Biolegend, REF# 149033), CCR2 (SA203G11, Biolegend, REF# 150605) and I-A /I-E (MS5/114.15.2 , Biolegend , REF# 107641). Samples were acquired on 13 color Cytoflex (Beckman Coulter) and analyses were performed with Kaluza software (Beckman Coulter).

**PDL-1 expression on RET cells by flow cytometry.** RET cells were grown and maintained in 6 wells plate (200 000 cells/ well) in the medium and conditions as described above. Cells were cultured with or without 10 ng/ mL of mouse recombinant IFNγ (rIFNg) (Peprotech) and 10mM 3HB (Ketoforce). After 48 hours, cells were harvested and stained with anti-PD-L1(CD274) (MIH5, ThermoFischer REF# 12-5982-82) Abs and isotype control Abs.

**GPR109 inhibition.** Mepenzolate bromide (C21H26BrNO3) (MPB), the pharmacological inhibitor of GPR109A was obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Starting the very day of RET challenge, the MPB was prepared and ip injected on a daily basis at 5 mg/ml until mouse sacrifice.

**BM-DC and Immortalized dendritic cells (iniDC).** The inducible immortalized dendritic cell (iniDC) line was a kind gift from Dr. Cornelia Richter (Technische Universitaet Dresden, Germany). Basic medium for the culture of iniDC is RPMI 1640 medium in supplement with fetal bovine serum (FBS, 10% v/v), penicillin (100 U/mL), streptomycin (100 μg/mL), 10 mM HEPES, 1 mM sodium pyruvate, and 50 μM β-Mercaptoethanol. Culture medium for the iniDCs was further supplied with 10 ng/mL GM-CSF (PeproTech Ref# 315-03). As previously published by Richter et al. iniDCs are immortalized under the induction of dexamethasone (Dex, purchased from Sigma, Ref# D0700000, final concentration at 100 nM) and doxycycline (Dox, purchased from Sigma, Ref# D3000000, final concentration at 2 μM). When Dex and
Dox are removed from the medium, which is defined the process “de-induction”, the iniDCs stop proliferate and differentiate into primary cell-like DCs, means the De-iniDC. These cells were incubated with increasing dosages of 3HB (Ketoforce) +/- 10 ng/ml of rIFNγ (Peprotech) and stained with anti-PD-L1(CD274) Abs and isotype control Abs (MIH5, REF# 12-5982-82). The same procedure was performed using BM-DC (bone marrow derived DC) cultivated from bone marrow precursors using recombinant GM-CSF+IL-4 as previously reported 49.

**Statistical analyses.** Data analyses and representations were performed either with the R software 65, Microsoft Excel (Microsoft Co., 436 Redmont, WA, US) or Prism 5 (GraphPad, San Diego, CA, USA. For group comparisons of mice, statistical analyses gathering more than two groups were performed using Kruskall-Wallis with Dunn's test to take into account multiple testing. For the log10-normalized data we performed ANOVA with Sidak's test (*p<0.05) and for two-way ANOVA, we perform ANOVA followed with Dunnet's test to take into account multiple testing. Otherwise, for two groups, statistical analyses were performed using the Mann-Whitney. Non-parametric tests were performed when the parametric assumptions did not hold (respectively Kruskall-Wallis test with post-hoc Dunn's test, and Mann-Whitney test). Outliers within a given distribution were tested using Grubbs’ test 66 with a threshold at p<0.05. All tumor growth curves were analyzed using software developed in Professor Guido Kroemer’s laboratory and information about statistical analyses can be found in references 67,68. p-values were two-sided with 95% confidence intervals and were considered significant when p<0.05. Symbol significance: *p<0.05, **p<0.01, ***p<0.001.

**Study approval.** All mice experiments were approved by the local institutional board and performed in accordance with government and institutional guidelines and regulations (APAFIS# 21378-201907080848483459).
Authors contributions:

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Conflict of interest:

LZ, RD and GK are founders of EverImmune.
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Figure Legends

**Figure 1. Ketogenic diet decreases melanoma and renal cell tumor growth.**

A-B. Growth kinetics of RET melanoma in C57BL/6 mice. The minimal tumorigenic dose of RET melanoma cells was inoculated subcutaneously the day of starting the dietary intervention [Normal diet (ND) vs ketogenic diet (KD)]. Tumor size was monitored three times a week for 12 days. Each curve represents the tumor kinetics of one animal (A) and the mean+SEM of tumor size was calculated for both nutritional interventions (B). The graphs depict the concatenation of 5 independent experiments comprising 5-6 mice/group (n=29-30 mice). C. Monitoring of RENCA progression using bioluminescence imaging of luciferase activity in four representative mice among 15 BALB/c mice fed ND versus KD. D. The ratio of luminescence at day 15 versus day 7 after orthotopic tumor injection was calculated for each diet (D15: IVIS measurement, D7: day of randomization). All experiments were composed of 5-7 mice/group and were performed at least twice, yielding similar results (n=14 for ND and n=12 for KD). Statistics: Dedicated software (https://kroemerlab.shinyapps.io/TumGrowth/) (A,B) and Student’s t-test *p<0.05 (D).

**Figure 2. Ketone bodies accumulate in tissues of ketogenic diet fed mice.**

A. Follow up of mouse weight over time until sacrifice in tumor bearers fed normal diet (ND) versus Ketogenic Diet (KD) (n= 30 for each groups). B-C. Heatmap of the non-supervised hierarchical clustering highlighting differences in the metabolic profiling of C57BL/6 mice fed ND (n=20) versus KD (n=21) in plasma (B) and PCA based on Bray Curtis Dissimilarity Index showing significant compositional differences across diet types [(C), PERMANOVA, p=0.001]. Arrows indicate 3-hydroxybutyrate (3HB) and acetoacetate positions in the heatmap. D-E. Concentrations of acetoacetate and 3HB in three compartments of tumor bearers. A representative experiment comprising 5 mice/group is depicted out of two
metabolomics profiles yielding similar results (n= 3 in ND and n=5 in KD). F. Spearman correlations between levels of 3HB in plasma and RET tumor size at day 12 indicating r and p values for the KD group. Not significant for the ND group. Statistics: ANOVA (A) & Student’s t-test statistical analyses of means+SEM: *p<0.05 (D,E), Spearman correlation (F).

Figure 3. Ketone body 3-hydroxybutyrate (3HB) is necessary and sufficient to account for the anticancer effects of ketogenic diet.

A,B. Similar experimental setting as in Figure 1A-B comparing RET tumor size at day 12 of each nutritional intervention (Normal diet (ND), ketogenic diet (KD), 3-hydroxybutyrate per os (3HB po) versus 3-hydroxybutyrate intra-peritoneal (3HB ip)) for all 12-15 mouse tumors from 3 independent experiments (A) and the complete regression rates for all the experiments performed (n, number of independent experiments encompassing 5-6 mice/group, SEM of % of tumor free mice across 3-7 experiments (B). C. Pharmacological inhibition of GPR109A (versus PBS as control) using intra-peritoneal daily administration of mepenzolate bromide (C21H26BrNO3). Tumor sizes at day 12 are depicted for each group. Concatenated data from 2-3 experiments comprising 5-6 mice/group are depicted (n=20 for ND, KD, 3HB po without mepenbrozolate and n=24 o ND, KD, 3HB po .D-F. Effects of sucrose supplementation on the antitumor (D) and metabolic effects (E,F) mediated by KD. Id. as in Fig. 1A-B showing RET tumor sizes at day 12 after various dietary interventions (D) and the two Ketones Bodies (KB) plasma metabolites as in Figure 2D-E (E,F). Results from one representative experiment out of 2 are depicted (n= 30 for group without sucrose and n=12 with sucrose groups).

Statistics: Global comparison using Kruskall-Wallis test, Post-hoc multiple comparisons using Dunn's test (A,B,C,D) and Student’s t-test (E and F) (*p<0.05, **p<0.01, ***p<0.001.)

Figure 4. Ketogenic diet shifts the microbiota composition.
A. PCoA representing the differences in beta-diversity of fecal microbiota between dietary interventions Normal diet (ND) versus Ketogenic diet (KD) at day 12 in RET tumor bearers mice. ANOSIM defines the separation of the groups; the p value defines the significance of such separation after 999 permutations of the samples. For each principal coordinate axis (PCo1 and PCo2), the collected variance, the Pearson Rho coefficient and the corresponding p value are shown in B. Partial Least Squares Discriminant Analysis (PLS-DA) plot of the variance between KD and ND fed tumor-bearing mice. LEfSe barplot of species at day 12 discriminating ND from KD fed tumor-bearing mice, ordered by their LDA score. Detailed relative abundance of distinct species in (C). Refer to Figure S4 for additional taxa. D. Effects of broad-spectrum antibiotics on the percentages of tumor-free mice after ND or KD or 3-hydroxybutyrate per os (3HB po) after RET inoculation. Concatenated data from 2-3 experiments comprising 5-6 mice/group are depicted (n=12 for ND group and n=21 for KD group). Statistics: Student t-test or ANOVA test. **p<0.01, ***p<0.001.

Figure 5. Correlations between microbial species and ketone bodies in mice and humans.

A-C. Mouse data. Alignment of the taxa relevant in our mouse preclinical studies and the plasma Ketone Bodies (KB) and Spearman correlations between two parameters. The significant ones are indicated with an asterisk (p<0.05) and their raw data are presented in B-C (each dot representing one mouse). D. Human data. Heatmap showing a correlation matrix between estimates of carbohydrate intake from Food Questionnary (FQ) and KB monitoring in plasma in >1,000 individuals from the PREDICT-1 study. The taxa highly significant in our mouse models are highlighted. Statistic: Pearson correlation.
Figure 6. T cell-dependent effects of ketogenic diet and synergy with immune checkpoint blockade.

A. Effects of T cell depletion on tumor sizes at day 12 of diet interventions. Tumor sizes in all diet [Normal diet (ND), Ketogenic diet (KD) or 3-hydroxybutyrate per os (3HB po)] fed groups in the presence of intraperitoneal (ip) injections of anti-CD4 and anti-CD8 depleting Abs prior to tumor inoculation. A representative experiment out of two is depicted, both yielding similar conclusions, each dot representing one mouse, each group containing 5-6 mice. Mann Whitney statistical analyses: *p<0.05, **p<0.01. B. Experimental setting for combinatorial regimen used in C-E. C-D. Effects of diet interventions on tumor size (C) at day 9 post-RET inoculation and diet intervention, after only one ip administration of cICB (anti-CTLA-4 + anti-PD-1 mAbs) and overall survival (D). Results of 4 concatenated experiments are depicted, each dot representing one mouse (n= 30 for ND, KD groups and n= 12 for 3HB po and 3HB ip). C. Fold increase of tumor sizes in cICB groups fed with ND, KD or 3HB po or 3-hydroxybutyrate intra-peritoneal (3HB ip) (normalized to ND groups). D. Overall survival appreciated with Kaplan Meier curves for 12 animals/group after a complete treatment combining continuous diet + cICB, accordingly to Fig 6B. E. Flow cytometry analyses of Tc1 cells (defined as CXCR3+CD8+ T splenocytes) at day 9 of the combinatorial regimen, each dot representing one spleen. Results from two pooled experiments are shown. F. Effects of sucrose supplementation on the synergy between diets and cICB against RET tumor progression. Individual tumor sizes at day 19 of three concatenated experiments comprising 6 mice/group are shown for each diet intervention, each dot representing one tumor/mouse (n=27 for groups without sucrose, n=28 for groups on sucrose). Statistics: Student’s t-test (A), Global comparison using Kruskall-Wallis test and post-hoc multiple comparisons using Dunn's test (C,E,F) (*p<0.05, **p<0.01, ***p<0.001.)
Figure 7. Intermittent 3-hydroxybutyrate scheduling affects systemic expression of T cell inhibitory receptor and their ligands.

A-B. Experimental setting for intermittent diet and combined Immune Checkpoint blockers (cICB) in the RET model and Kaplan Meier curves of overall survival for 12 animals/group, according to therapeutic scheme comparing continuous (Cont) versus intermittent (On/off) diet interventions. C. Flow cytometric analyses of PD-1 and CTLA-4 expression in splenic CD8 T lymphocytes at day 17, after diet (Ketogenic diet (KD) or 3-hydroxybutyrate (3HB) Cont or On/off) and systemic cICB combinatorial regimen in RENCA bearing BALB/c mice. Refer to Figure S5A-B for Tim-3, Lag-3 and 4-1BB in all splenic T cells. D-E. Flow cytometry analysis of MFI for the membrane expression of costimulatory (CD86 in D) or inhibitory (PD-L1 in E) on macrophages (D) and other myeloid subsets (D,E) from the spleens at day 17, after complete diet (3HB On/off) and systemic cICB combinatorial regimen in RENCA bearing BALB/c mice. The results from 2-3 experiments comprising 6 mice/group are depicted, each dot representing one spleen. F,G. In vitro effects of increasing dosing of 3HB onto a Dendritic Cell line (DC cell line) stimulated or not with rIFNg. Flow cytometry determination of MHCII/I-Ab (F) and PD-L1 expression (G) as mean fluorescence intensity (MFI) on the surface expression at 48 hours of stimulation. A representative experiment out of 4 is depicted, yielding similar conclusions (n= 12 or each groups). Statistics: Student’s t-test (C), Global comparison using Kruskall-Wallis test, Post-hoc multiple comparisons using Dunn's test (*p<0.05) (D and E). Global comparison using ANOVA, Post-hoc multiple comparisons using Dunnet's test"(*p<0.05) (F and G) (*p<0.05, **p<0.01, ***p<0.001).

Figure 8. Efficacy of intermittent 3-hydroxybutyrate in circumventing primary resistance to PD-1 blockade in an orthotopic cancer model.
A-F. Extension of the comparison between continuous (Cont) versus intermittent (On/off) feeding with KD or 3HB po in two tumor models [RENCA (A), TC-1 (B-D)] treated with various therapeutic mAbs (refer to Figure S6A and B for experimental setting designs). Cross-sectional RENCA tumor burden monitored by bioluminescence imaging of luciferase activity [ratio of luminescence at day 8 versus day 0 (D8: day of randomization). (A) are depicted, gathering two independent experiments containing 6 mice/group. All experiments were performed twice. Monitoring of TC-1 progression using bioluminescence imaging (B) and percentages of TC-1-luc tumor-free animals overtime monitored by bioluminescence imaging of luciferase activity (C) and overall survival (D) estimated by Kaplan Meier curves for 2 experiments of 6 mice/group pooled together. E,F. Tumor growth curves represented as means+SEM of tumor sizes for each group after subcutaneous re-challenge of TC-1-luc tumor-free mice (from B-D) with the MTD of TC-1-luc (E) or irrelevant MCA205 (F) (refer to Figure S6C for experimental setting designs). Experiments were performed twice (n=12 per groups). Statistics: Global comparison for the log10-normalized data using ANOVA, Post-hoc multiple comparisons using Sidak's test (*p<0.05) (A) or dedicated software (https://kroemerlab.shinyapps.io/TumGrowth/) (C, D, E, F). *p<0.05, **p<0.01, ***p<0.001.

Table 1. List of metabolites differentially monitored in plasma of ketogenic diet-fed animals versus normal diet-fed littermates.

Plasma metabolites significantly increased with ND (Cluster A), or with KD (cluster C) or unchanged (middle columns) are listed, according to the heatmap and non-hierarchical clustering depicted in Figure 2B.
Figure 1. Ketogenic diet decreases melanoma and renal cell tumor growth.

A-B. Growth kinetics of RET melanoma in C57BL/6 mice. The minimal tumorigenic dose of RET melanoma cells was inoculated subcutaneously the day of starting the dietary intervention [Normal diet (ND) vs ketogenic diet (KD)]. Tumor size was monitored three times a week for 12 days. Each curve represents the tumor kinetics of one animal (A) and the mean+SEM of tumor size was calculated for both nutritional interventions (B). The graphs depict the concatenation of 5 independent experiments comprising 5-6 mice/group (n=29-30 mice). C. Monitoring of RENCA progression using bioluminescence imaging of luciferase activity in four representative mice among 15 BALB/c mice fed ND versus KD. D. The ratio of luminescence at day 15 versus day 7 after orthotopic tumor injection was calculated for each diet (D15: IVIS measurement, D7: day of randomization). All experiments were composed of 5-7 mice/group and were performed at least twice, yielding similar results (n=14 for ND and n=12 for KD). Statistics: Dedicated software (https://kroemerlab.shinyapps.io/TumGrowth/) (A,B) and Student’s t-test *p<0.05 (D).
Figure 2. Ketone bodies accumulate in tissues of ketogenic diet fed mice.

A. Follow up of mouse weight over time until sacrifice in tumor bearers fed normal diet (ND) versus Ketogenic Diet (KD) (n= 30 for each groups). B–C. Heatmap of the non-supervised hierarchical clustering highlighting differences in the metabolic profiling of C57BL/6 mice fed ND (n=20) versus KD (n=21) in plasma (B) and PCA based on Bray Curtis Dissimilarity Index showing significant compositional differences across diet types [(C), PERMANOVA, p=0.001]. Arrows indicate 3-hydroxybutyrate (3HB) and acetoacetate positions in the heatmap. D–E. Concentrations of acetoacetate and 3HB in three compartments of tumor bearers. A representative experiment comprising 5 mice/group is depicted out of two metabolomics profiles yielding similar results (n= 3 in ND and n=5 in KD). F. Spearman correlations between levels of 3HB in plasma and RET tumor size at day 12 indicating r and p values for the KD group. Not significant for the ND group. Statistics: ANOVA (A) & Student’s t-test statistical analyses of means+SEM: *p<0.05 (D,E), Spearman correlation (F).
Figure 3. Ketone body 3-hydroxybutyrate (3HB) is necessary and sufficient to account for the anticancer effects of ketogenic diet.

A,B. Similar experimental setting as in Figure 1A-B comparing RET tumor size at day 12 of each nutritional intervention (Normal diet (ND), ketogenic diet (KD), 3-hydroxybutyrate per os (3HB po) versus 3-hydroxybutyrate intra-peritoneal (3HB ip)) for all 12-15 mouse tumors from 3 independent experiments (A) and the complete regression rates for all the experiments performed (n, number of independent experiments encompassing 5-6 mice/group, SEM of % of tumor free mice across 3-7 experiments (B). C. Pharmacological inhibition of GPR109A (versus PBS as control) using intra-peritoneal daily administration of mepenzolate bromide (C21H26BrNO3). Tumor sizes at day 12 are depicted for each group. Concatenated data from 2-3 experiments comprising 5-6 mice/group are depicted (n=20 for ND, KD, 3HB po without mepenzbrozolate and n=24 o ND, KD, 3HB po .D-F. Effects of sucrose supplementation on the antitumor (D) and metabolic effects (E,F) mediated by KD. Id. as in Fig. 1A-B showing RET tumor sizes at day 12 after various dietary interventions (D) and the two Ketones Bodies (KB) plasma metabolites as in Figure 2D-E (E,F). Results from one representative experiment out of 2 are depicted (n= 30 for group without sucrose and n=12 with sucrose groups). Statistics: Global comparison using Kruskall-Wallis test, Post-hoc multiple comparisons using Dunn's test (A,B,C,D) and Student's t-test (E and F) (**p<0.01, ***p<0.001.)
Figure 4. Ketogenic diet shifts the microbiota composition.

A. PCoA representing the differences in beta-diversity of fecal microbiota between dietary interventions Normal diet (ND) versus Ketogenic diet (KD) at day 12 in RET tumor bearers mice. ANOSIM defines the separation of the groups; the p value defines the significance of such separation after 999 permutations of the samples. For each principal coordinate axis (PCo1 and PCo2), the collected variance, the Pearson Rho coefficient and the corresponding p value are shown in B. Partial Least Squares Discriminant Analysis (PLS-DA) plot of the variance between KD and ND fed tumor-bearing mice. LEfSe barplot of species at day 12 discriminating ND from KD fed tumor-bearing mice, ordered by their LDA score. Detailed relative abundance of distinct species in (C). Refer to Figure S4 for additional taxa. D. Effects of broad-spectrum antibiotics on the percentages of tumor-free mice after ND or KD or 3-hydroxybutyrate per os (3HB po) after RET inoculation. Concatenated data from 2-3 experiments comprising 5-6 mice/group are depicted (n=12 for ND group and n=21 for KD group). Statistics: Student t-test or ANOVA test. **p<0.01, ***p<0.001.
Figure 5. Correlations between microbial species and ketone bodies in mice and humans.

A-C. Mouse data. Alignment of the taxa relevant in our mouse preclinical studies and the plasma Ketone Bodies (KB) and Spearman correlations between two parameters. The significant ones are indicated with an asterisk (p<0.05) and their raw data are presented in B-C (each dot representing one mouse). D. Human data. Heatmap showing a correlation matrix between estimates of carbohydrate intake from Food Questionnary (FQ) and KB monitoring in plasma in >1,000 individuals from the PREDICT-1 study. The taxa highly significant in our mouse models are highlighted. Statistic: Pearson correlation.
Figure 6. T cell-dependent effects of ketogenic diet and synergy with immune checkpoint blockade.

A. Effects of T cell depletion on tumor sizes at day 12 of diet interventions. Tumor sizes in all diet [Normal diet (ND), Ketogenic diet (KD) or 3-hydroxybutyrate per os (3HB po)] fed groups in the presence of iintraperitoneal (ip) injections of anti-CD4 and anti-CD8 depleting Abs prior to tumor inoculation. A representative experiment out of two is depicted, both yielding similar conclusions, each dot representing one mouse, each group containing 5-6 mice. Mann Whitney statistical analyses: *p<0.05, **p<0.01. B. Experimental setting for combinatorial regimen used in C-E. C-D. Effects of diet interventions on tumor size (C) at day 9 post-RET inoculation and diet intervention, after only one ip administration of clCB (anti-CTLA-4 + anti-PD-1 mAbs) and overall survival (D). Results of 4 concatenated experiments are depicted, each dot representing one mouse (n= 30 for ND, KD groups and n= 12 for 3HB po and 3HB ip). C. Fold increase of tumor sizes in clCB groups fed with ND, KD or 3HB po or 3-hydroxybutyrate intraperitoneal (3HB ip) (normalized to ND groups). D. Overall survival appreciated with Kaplan Meier curves for 12 animals/group after a complete treatment combining continuous diet + clCB, accordingly to Fig 6B. E. Flow cytometry analyses of Tc1 cells (defined as CXCR3+CD8+ T splenocytes) at day 9 of the combinatorial regimen, each dot representing one spleen. Results from two pooled experiments are shown. F. Effects of sucrose supplementation on the synergy between diets and clCB against RET tumor progression. Individual tumor sizes at day 19 of three concatenated experiments comprising 6 mice/group are shown for each diet intervention, each dot representing one tumor/mouse (n=27 for groups without sucrose, n=28 for groups on sucrose). Statistics: Student’s t-test (C), Global comparison using Kruskall-Wallis test, Post-hoc multiple comparisons using Dunn’s test (*p<0.05) (D and E). Global comparison using ANOVA, Post-hoc multiple comparisons using Dunnett’s test" (*p<0.05) (F and G) (*p<0.05, **p<0.01, ***p<0.001).
Figure 7. Intermittent 3-hydroxybutyrate scheduling affects systemic expression of T cell inhibitory receptor and their ligands.

A-B. Experimental setting for intermittent diet and combined immune checkpoint blockers (clCB) in the RET model and Kaplan Meier curves of overall survival for 12 animals/group, according to therapeutic scheme comparing continuous (Cont) versus intermittent (On/off) diet interventions. C. Flow cytometric analyses of PD-1 and CTLA-4 expression in splenic CD8 T lymphocytes at day 17, after diet (Ketogenic diet (KD) or 3-hydroxybutyrate (3HB) Cont or On/off) and systemic clCB combinatorial regimen in RENCA bearing BALB/c mice. Refer to Figure S5A-B for Tim-3, Lag-3 and 4-1BB in all splenic T cells. D-E. Flow cytometry analysis of MFI for the membrane expression of costimulatory (CD86 in D) or inhibitory (PD-L1 in E) on macrophages (D) and other myeloid subsets (D,E) from the spleens at day 17, after complete diet (3HB On/off) and systemic clCB combinatorial regimen in RENCA bearing BALB/c mice. The results from 2-3 experiments comprising 6 mice/group are depicted, each dot representing one spleen. F,G. In vitro effects of increasing dosing of 3HB onto a Dendritic Cell line (DC cell line) stimulated or not with rIFNg. Flow cytometry determination of MHCII/l-Ab (F) and PD-L1 expression (G) as mean fluorescence intensity (MFI) on the surface expression at 48 hours of stimulation. A representative experiment out of 4 is depicted, yielding similar conclusions (n= 12 or each groups). Statistics: Student’s t-test (C), Global comparison using Kruskall-Wallis test, Post-hoc multiple comparisons using Dunn’s test (*p<0.05) (D and E). Global comparison using ANOVA, Post-hoc multiple comparisons using Dunnet’s test (“*p<0.05) (F and G) (“*p<0.05, **p<0.01, ***p<0.001).
Figure 8. Efficacy of intermittent 3-hydroxybutyrate in circumventing primary resistance to PD-1 blockade in an orthotopic cancer model.

A-F. Extension of the comparison between continuous (Cont) versus intermittent (On/off) feeding with KD or 3HB po in two tumor models [RENCA (A), TC-1 (B-D)] treated with various therapeutic mAbs (refer to Figure S6A and B for experimental setting designs). Cross-sectional RENCA tumor burden monitored by bioluminescence imaging of luciferase activity [ratio of luminescence at day 8 versus day 0 (D8: day of randomization). (A) are depicted, gathering two independent experiments containing 6 mice/group. All experiments were performed twice. Monitoring of TC-1 progression using bioluminescence imaging (B) and percentages of TC-1-luc tumor-free animals overtime monitored by bioluminescence imaging of luciferase activity (C) and overall survival (D) estimated by Kaplan Meier curves for 2 experiments of 6 mice/group pooled together. E,F. Tumor growth curves represented as means±SEM of tumor sizes for each group after subcutaneous re-challenge of TC-1-luc tumor-free mice from B-D with the maximum tolerated dose of TC-1-luc (E) or irrelevant MCA205 (F) (refer to Figure S6C for experimental setting designs). Experiments were performed twice (n=12 per groups). Statistics: Global comparison for the log10-normalized data using ANOVA, Post-hoc multiple comparisons using Sidak's test (*p<0.05) (A) or dedicated software (https://kroemerlab.shinyapps.io/TumGrowth/) (C, D, E, F). *p<0.05, **p<0.01, ***p<0.001.
Table 1. List of metabolites differentially monitored in plasma of ketogenic diet-fed animals versus normal diet-fed littermates.

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Table 1. List of metabolites differentially monitored in plasma of ketogenic diet-fed animals versus normal diet-fed littermates.

This table refers to the non-supervised hierarchical clustering of metabolites of C57BL/6 mice fed normal diet (ND) versus ketogenic diet (KD) in plasma. (Data from Figure 2B) Metabolites increased in ND are in Cluster A (in green, left column), metabolites increased in KD are in Cluster C (in red, right column) Cluster B are metabolites with few modification or unchanged (in black, middle column).

ND = Normal Diet, KD = Ketogenic Diet