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Original Article

The effect of redox bacteria on the programmed cell death-1 cancer immunotherapy

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Abstract

Background and purpose: Extracellular electron transferring (EET) or redox bacteria employ a shuttle of flavins to transfer electrons to the oxygen in the intestinal mucosa. Although clinical studies suggest that the gut microbiome modulates the efficiency of immune checkpoint therapy in patients with cancer, the modulation mechanisms have not been well-characterized yet.
Experimental approach: In the present study, the oral gavage administration of *Shewanella oneidensis* MR-1 as a prototypic EET bacteria was assayed in a mouse model of lung cancer to determine the effect of EET bacterium on the efficacy of the programmed cell death protein 1 (PD1)-immune checkpoint therapy.
Findings/Results: It was indicated that *in vitro* EET from *S. oneidensis* was mediated by riboflavins that were supplied through extrinsic sources. Co-administration of *S. oneidensis* and anti-PD1 antibodies represent better tumor remission compared to the single-administration of each one; however, no statistically significant change was observed in the tumor volume.
Conclusion and implications: More detailed studies are needed to definitively confirm the therapeutic effects of electrogenic bacteria in patients with cancer. Given the findings of the present study, increasing flavin compounds or EET bacteria in the intestine may provide novel strategies for modulating cancer immunotherapy.

Keywords: Cancer immunotherapy; Electrogenic bacteria; Programmed cell death protein-1.

INTRODUCTION

Immune checkpoint therapy (ICT) strategies increase T lymphocyte-mediated responses against cancer cells by blocking the interaction of inhibitory receptors of T cells with their counterpart ligands (1). Monoclonal antibodies (mAbs) inhibiting the interaction of programmed cell death protein 1 (PD1) with its ligand PD-L1 are widely used in ICTs (2). New studies have highlighted the effective role of the gut microbiome in tumor therapeutic responses to chemotherapy and ICTs (3,4). Several models discuss mechanisms for the influence of the microbiome on antitumor immunosurveillance (5), particularly for explaining the relationship between gut microbiota and the efficiency of PD1 cancer immunotherapies (3).

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In some studies, it was found that some biochemical compounds in the ICT-responder patients were different from the non-responder group (6,7). Analysis of the blood metabolites from patients with non-small cell lung cancer before and after treatment with anti-PD1 therapy showed that metabolites derived from the microbiome and redox metabolites such as cysteine and glutathione disulfide provide a high response probability (8). In addition, oral supplementation with *Akkermansia muciniphila* restores the efficacy of PD1 antibody therapy by inducing dendritic cells to secret interleukin-12 (IL-12) (9) which polarizes naïve T cells into Th1 cells that are efficient immune cells against tumor cells.

The demonstrated association among factors, including the redox potential, the intestinal microbiome, and the effectiveness of the PD1 therapy, prompted us to develop an experiment that aimed to examine the direct effect of externally induced redox potential by intestinal bacteria on the effectiveness of PD1 therapy in a mouse model of non-small cell lung cancer. In this study, the relationship between redox potential changes in the intestine and the therapeutic effect of the ICT was investigated using the colonization of bacteria, that could transfer electrons out of the cell, and the administration of an anti-PD1 antibody.

Living organisms require energy for their function and proliferation through processes such as respiration, photosynthesis, or fermentation. These processes are fundamentally based on oxidation and reduction and the involvement of electron transfer reactions. Most eukaryotic and prokaryotic cells conduct oxidation and reduction of chemical compounds inside their cells. However, some microorganisms can transfer electrons to or from extracellular solid materials to acquire cellular energy, which is called extracellular electron transfer (EET) (10).

Four types of endogenous bioelectric were defined in the body, including (a) maintaining voltage potentials across the membranes of subcellular organelles, (b) the function of ion channels and pumps maintaining cell plasma membranes potential (V_{mem}), (c) transepithelial potential (V_{TEP}) derived from cells organized into tissues, and (d) the electricity of entire anatomical body axes (11).

In a position of aerobic glycolysis, cancer cells reprogram energy metabolism to the Warburg effect, which is characterized by low levels of oxidative phosphorylation and upregulated glycolysis. An increased rate of glycolysis leads to an increased level of reduced electrochemical mediators such as NADH (12). The oxidation of these reduced electrochemical mediators is essential for the continuous flux of glucose through glycolysis. There are two mechanisms in the plasma membrane to oxidize cytosolic NADH, including the function of NADH oxidase, which uses oxygen as a terminal electron acceptor, and the function of a protein complex called porin or voltage-dependent anion channel (VDAC), which transfers an electron across the membrane on an SH group (13). Biochemical entities, such as NADH, ascorbic acid, and ubiquinone, can rapidly accept and donate electrons, leading to the faradaic current (12).

The association between the gut microbiota and the results of ICTs has been reported in patients suffering from cancers, but the mechanisms underlying this phenomenon remain unknown. The hypothesis proposed in this article is whether the alteration in the redox potential or electrogenesis of the gut microbiome would change the therapeutic results of PD1 blockade antibodies. TC-1, which is a murine epithelial cell line and constitutively expresses PD-L1 (14) was used for tumor induction in mice. This cell line is widely used in pre-clinical studies of mouse models of PD1 blockade therapy (15).

MATERIALS AND METHODS

Bacterial culture and colony counting

Shewanella oneidensis strain number IBRC-M 10991 was purchased from the Iranian Biological Resource Center (IBRC) and cultured in Luria-Bertani (LB) broth media at 37 °C in an aerobic tent. In this study, a non-pathogenic strain of *Escherichia coli* (BL-21) was used as control bacteria and it was also cultured in LB broth media. To colony count, serial dilution of bacteria (1/10, 1/100, 1/1000, *etc*.) was made in l mL of LB broth media and

then cultivated in dishes containing LB agar media at 37 °C in an aerobic tent. After an overnight incubation period, the colony-forming units (CFUs) in the dishes were visually counted for each bacteria strain. To this end, CFU was calculated by multiplying bacterial colonies by the dilution used on the plate. Cultures were resuspended in LB broth media containing 15% glycerol and stored at -20 °C up to use for oral gavage administration.

EET assay

In this study, for the first time, we developed a new method for evaluating the redox potential or EET in bacteria and mammalian cell cultures. To test methylene blue dye reduction, 100 µL of 1.3 mM methylene blue dye prepared in potassium phosphate buffer solution (50 mM, pH 7.5) was added to 50 mL of the reaction mixture containing 60 µM $CaCl_2$, 36 µM $MgCl_2$, 15 mM NaCl, 3.3 mM sodium acetate. In addition, 50 mM potassium phosphate buffer was utilized as the diluent for solving agents of the reaction mixture. Thirty µL of reaction mixture containing methylene blue and 15 µL riboflavin (10 mg/mL) were added to the serial dilutions of *S. oneidensis* that were prepared in a liquid medium. After a 3-h incubation at 37 °C, the optical densities (ODs) at 450 nm were measured using a Unico 2100 spectrophotometer (United States of America). The same condition was used for the EET assay in the control bacteria, and the initial 600 nm OD was considered 0.4 for both *S. oneidensis* and control bacteria.

Methylene blue dye reduction assay for tumor and normal human cells

TC-1, a tumor cell line derived from lung epithelial cells of C57BL/6 mice, was purchased from the Pasteur Institute of Iran. Cells were grown in RPMI 1640 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 U/mL streptomycin/penicillin, 1 mM sodium pyruvate, 2 mM nonessential amino acids, and cultured at 37 °C in a humidified atmosphere with 5% CO_2. For normal control cells, peripheral blood mononuclear cells (PBMCs) were isolated from a healthy donor using a Ficoll density gradient. Thirty µL reaction mixture containing methylene blue and 15 µL riboflavin (10 mg/mL) were added to a different number of cells in a tissue culture plate. Phorbol 12-myristate 13-acetate (PMA) was added to the PBMC culture to obtain activated cells. The decolorization of cell culture supernatants was measured after a 3-h incubation at 37 °C in a humidified atmosphere with 5% CO_2, using a Unico 2100 spectrophotometer.

Animals and tumor model

All animal studies were approved by the Animal Care and Use Committee at Kashan University of Medical Sciences (Ethic No. IR.KAUMS.NUHEPM.REC.1399.010).

C57Bl6 inbred mice, 8-12 weeks of age, were purchased from the laboratory animal core facility of Pasteur Institute (Karaj, Iran). Mice were transported in specialized shipping cages and were housed at the core facility of laboratory animals, Kashan University of Medical Sciences. Mice were implanted with 1×10^6 TC-1 cells subcutaneously. Ten days after tumor implantation, microbiota colonization of mice was performed by oral gavage administration of 200 µL of suspension containing 1×10^9 bacteria. Mice were treated intraperitoneally with anti-PD1 mAb (40 µg/mouse; clone 1-14) and bacterial gavage three times per week. Tumor size was monitored every two days employing a caliper.

RNA isolation and cDNA synthesis

Mice were sacrificed with inhalation of diethyl ether in a homemade anesthesia chamber. The colon was placed in the RNX^+ (SinaClone, Iran) liquid of the RNA extraction kit. Tissues were homogenized and then RNA was extracted according to the manufacturer's protocol. Total RNA was reverse transcribed using the Pars Tous commercial kit (Pars Tous, Iran) according to the manufacturer's instructions. Briefly, 2 µL of extracted RNA was combined with 10 µL of 2× buffer mix, 2 µL enzyme mix, and 8 µL diethylpyrocarbonate (DEPC)-treated water to adjust the total volume to 20 µL. The sample was mixed with a quick vortex and incubated first at 25 °C for 10 min, second at 47 °C for 60 min, and finally at 85 °C for 5 min.

Quantitation of gene expression by real-time PCR

Primer's sequence of IL-12 forward; 5'-AAATGAAGCTCTGCATCCTGC-3' and reverse; 5'-TCACCCTGTTGATGGTCACG-3' were synthesized by Metabion (Munich, Germany). To standardize the relative assays, glycerol aldehyde phosphate dehydrogenase (GAPDH) forward primer; 5'-AGGTCGGTGTGAACGGATTTG-3' and reverse primer; 5'-TGTAGACCATG-TAGTTGAGGTCA-3' were determined as an internal control. The real-time polymerase chain reaction (RT-PCR) mixture consisted of 2 µL cDNA, 1 µL of each reverse and forward primer, 10 µL real-time master mix (Amplicon, Denmark), and 6 µL double-distilled water (ddH_2O) for a final volume of 20 µL. RT-PCR was carried out using an ABI thermocycler (Thermo Fisher, United States). The resulting changes in the fluorescence of amplification were relatively measured using Step-One V2.3 software.

Statistical analysis

Data, expressed as means ± SEM, were analyzed using one-way analysis of variance (ANOVA) followed by Tukey post-hoc test and the Student's t-test wherever required using GraphPad Prism 5. The *P*-values < 0.05 were considered statistically significant.

RESULTS

Riboflavin mediates the EET of S. oneidensis to methylene blue dye

Previous tests indicated that riboflavin is essential in the extracellular electron shuttling of *Faecalibacterium prausnitzii* (16). Color changes were significant in the methylene blue dye reduction assay when riboflavin was added to a tube containing different amounts of bacterial media. Importantly, no significant color changes were observed when riboflavin was added to tubes containing control bacteria (Fig. 1A). These findings showed unambiguously that *S. oneidensis* but not for BL-21 changed the color of methylene blue dyes in the presence of riboflavin. In this experiment, it was found that methylene blue discoloration due to the electron transfer from *S. oneidensis* was mediated by riboflavin (Fig. 1B), and this discoloration was clearly identified at different volumes of bacterial culture.

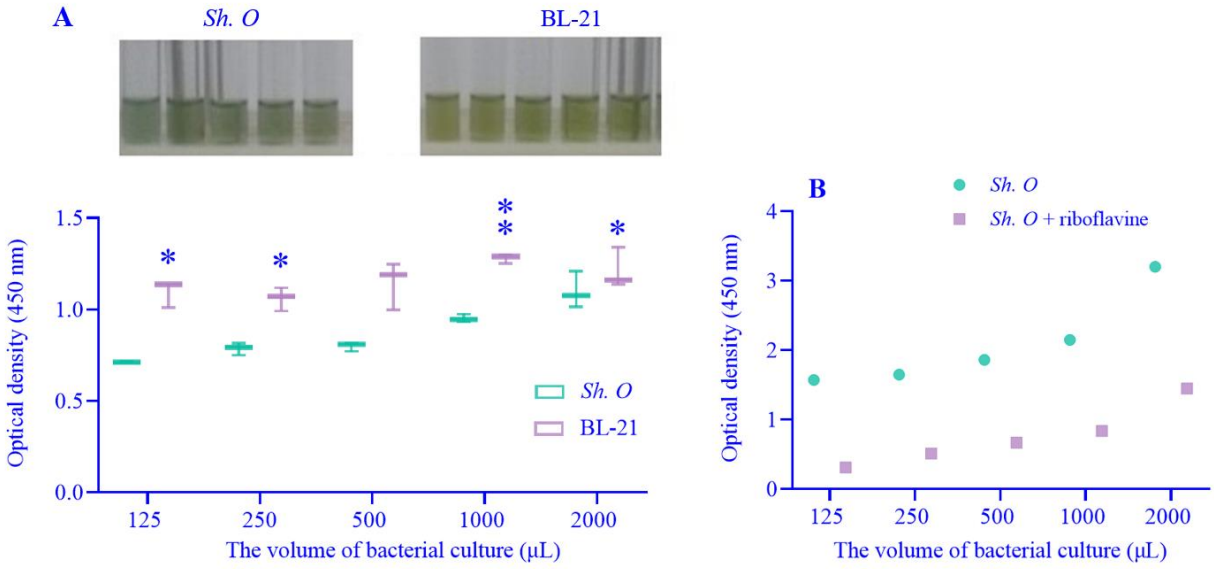

Fig. 1. Methylene blue reduction assay for electrogenic and non-electrogenic bacteria. (A) The redox indicator (reaction mixture containing methylene blue and riboflavin) was added to different concentrations of growing *Sh. O* and (B) BL-21 bacteria, n = 3; (B) The redox indicator with and without riboflavin was added to different concentrations of growing *Sh. O*. *$P \leq 0.05$ and **$P \leq 0.01$ indicate the significant differences between the two groups of each volume of bacterial culture. *Sh. O, Shewanella oneidensis*.

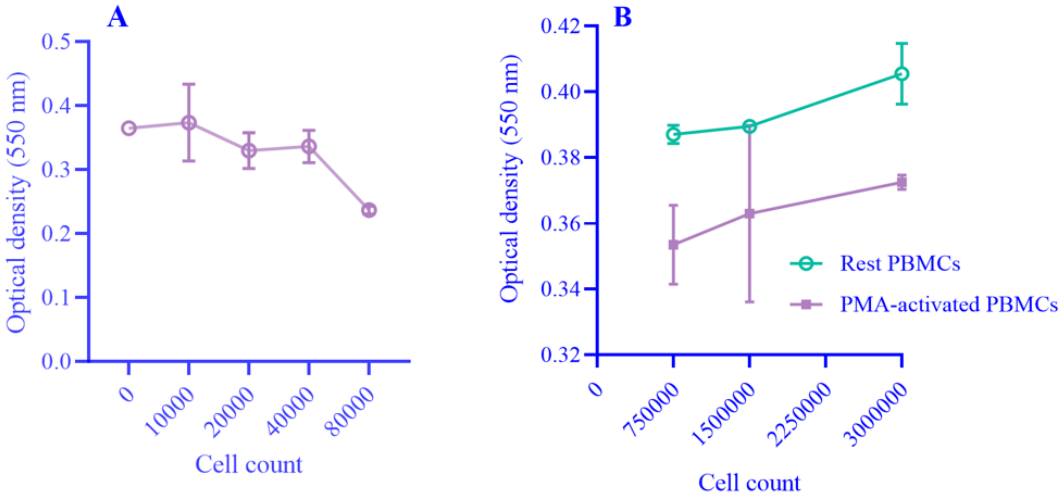

Fig. 2. Methylene blue reduction assay for proliferative and rest cells. (A) the redox indicator was also used for the assessment of the redox potential in different counts of growing epithelial tumor cells (TC-1); (B) the methylene blue redox indicator was used to determine the redox potential of different counts of resting PBMCs and activated PBMCs using a mitogen (PMA). PBMC, Peripheral blood mononuclear cells; PMA; phorbol myristate acetate.

Activated PBMCs and tumor cells reduce methylene blue absorbance.

To test the Warburg effect in cancer and activated cells modulate tumor microenvironment redox potential, we evaluated methylene blue absorbance change in the TC-1 cell culture. As shown in Fig. 2A, color changes were significant when cell numbers were increased. This finding showed that cancer cells use riboflavin to transfer an electron to methylene blue. Methylene blue staining is widely used to differentiate between yeast-viable and dead cells (17). The test was performed on PBMCs and PMA-activated PBMCs to assess redox potential in activated and rest leukocytes. The methylene blue color changes in the PMA-activated cells were significantly different from the color changes in the rest cells (Fig. 2B). This finding was compatible with the metabolic pathway used in the activated cells. The Warburg effect is the master metabolic pathway used in the activated cells.

The assessment of co-administration of PD1 blockade antibodies and electrogenic bacteria in a mouse tumor model

To test the effect of redox potential in the gut microbiome on the therapeutic efficiency of immune checkpoint therapies, we evaluated the gut colonization of *S. oneidensis* on the anti-PD1 therapy in tumor-implanted mice. In the assayed hypothesis, three main sources of electrons in the cancer patient are suggested, including electrons from the Warburg effect in activated leukocytes and tumor cells and gut electrogenic microbiome. Tumor size decreased in mice treated with *S. oneidensis* and this decrease was augmented by anti-PD1 antibodies (Fig. 3).

IL-12 expression assay in the colon of treated and control mice

Relative expression of the IL-12 gene was assayed for different groups of the study in comparison with the group of healthy mice that received no antibodies or bacteria (Fig. 4). According to the obtained results, no significant difference was observed between IL-12 gene expression in the control group and other groups. However, there was a significant difference in gene expression between the group receiving BL-21 bacteria and other groups, including cancer, the group receiving BL-21 bacteria and anti-PD1 antibodies, and the group receiving Sh. O bacteria.

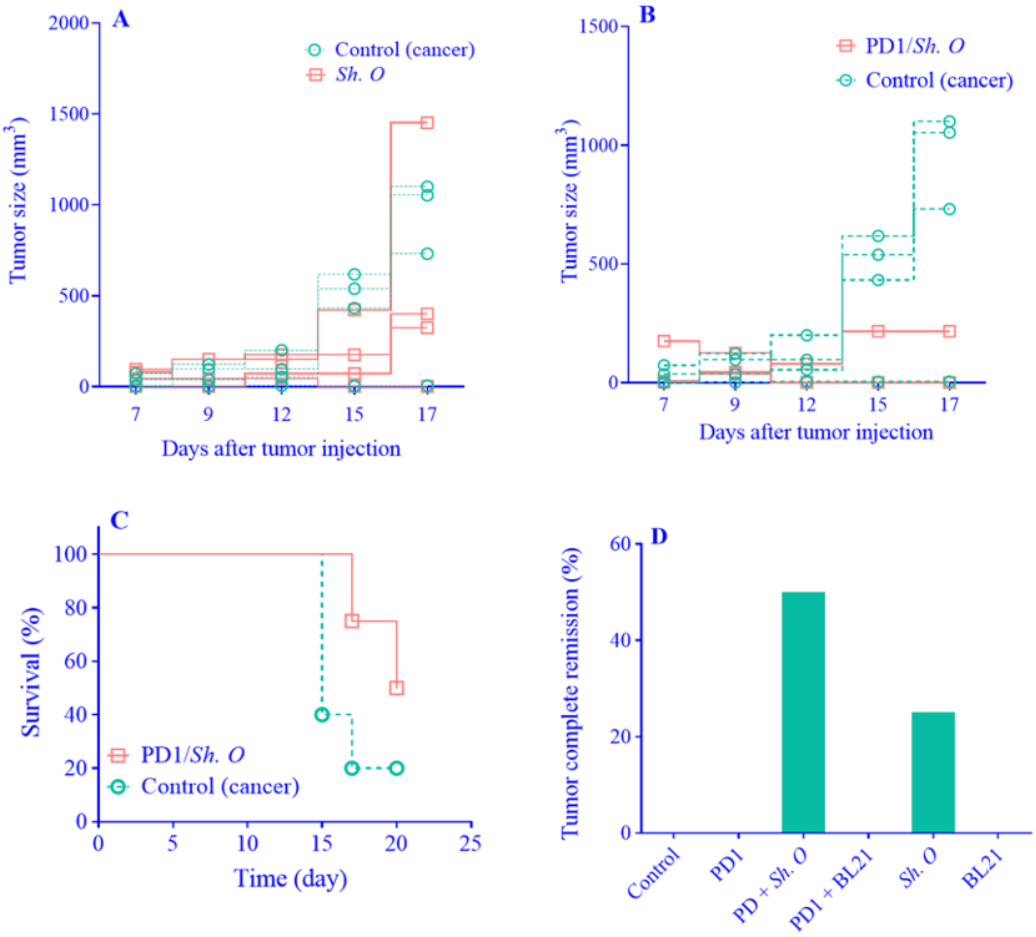

Fig. 3. The assessment of co-administration of PD1 blockade antibodies and electrogenic bacteria in a mouse tumor model. (A) TC-1 tumor growth in mice was orally gavaged with *Sh. O* and the control mice without receiving bacteria; (B) TC-1 tumor growth in mice co-administrated with oral gavage of *Sh. O* and intraperitoneal injection of PD1 blockade antibodies and the control mice; (C) survival curve in mice co-administrated with *Sh. O* and PD1 blockade antibodies and control mice; (D) the percentage of tumor complete remission in mice orally gavaged by BL-21 (BL-21), intraperitoneally injected with PD1 blockade antibodies (PD1), co-administration of orally gavaged *Sh. O* and PD1 blockade antibodies (PD1; *Sh. O*), co-administration of orally gavaged BL-21 and PD1 blockade antibodies (PD1; BL-21); n = 3-5. PD1, programmed cell death protein 1; BL-21, the non-pathogenic strain of *Escherichia coli; Sh. O, Shewanella oneidensis.*

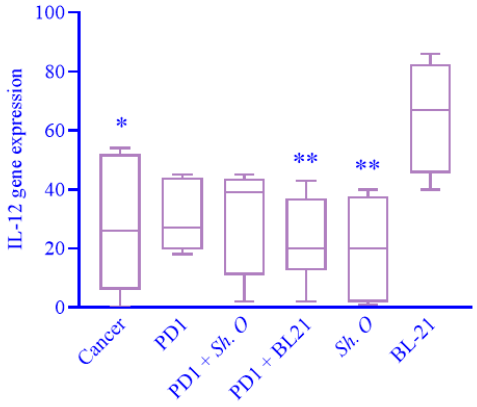

Fig. 4. Relative gene expression of IL-12 in the colon. The comparison of the relative expression of the IL-12 gene in different groups of the study in comparison with the group of healthy mice that received no antibodies or bacteria (control) revealed no significant differences. The groups include cancer: tumor-implanted mice, PD1, PD1 + *Sh. O*, PD1 + BL-21, *Sh. O*, and BL-21: tumor-implanted mice that received these interventions: PD1 blockade antibodies intraperitoneally; PD1 blockade antibodies and oral gavage of *Sh. O* bacteria; PD1 blockade antibodies and BL-21 bacteria; *Sh. O* bacteria; BL-21 bacteria. *$P \leq 0.05$ and **$P \leq 0.01$ indicate the significant differences compared to the BL-21 group. IL, Interleukin; PD1, programmed cell death protein 1; BL-21, a non-pathogenic strain of *Escherichia coli; Sh. O, Shewanella oneidensis.*

DISCUSSION

Several studies have reported that the gut microbiome influences the efficiency of PD1 blockade immunotherapy. In these studies, several bacterial strains and metabolites were discovered in responder and non-responder patients to PD1 blockade antibodies (3). Although various hypotheses have been raised to interpret the therapeutic effects of the microbiome (5), due to issues such as the complexity of the intestinal microbiome and immune system responses as well as the behavior of cancer cells, an exact mechanism for this therapeutic effect is still unknown.

In this study, for the first time, we investigated the effect of changes in the redox potential of the intestinal microbiome on the therapeutic effect of a biological substance. We aimed to determine the possible role of changes in the redox potential of the intestinal microbial flora on the therapeutic effect of PD1 blockade antibodies in the treatment of cancer. Initially, for the first time, a facile method was developed to detect the EET in biological media such as bacterial and mammalian cell cultures. The double-chamber cell or microbial fuel cell (MFC) is widely used for the electrochemical evaluation of EET in biological media (18,19). In this study, instead of microbial fuel cells, the discoloration of methylene blue in the presence of riboflavin was used to assay the redox potential of cell culture media. No need for microbial fuel cell equipment and the accessibility of the colorimetric method is the advantage of this method.

It seems that free riboflavin in the reaction mixture is required for electron transfer from *S. oneidensis* to the redox indicator and possibly to the other electron acceptors. Riboflavin, present in yeast extract, was reported to be exploited by the fermentative bacterium *Lactococcus lactis*, as an exogenous redox mediator for extracellular electron transfer (20). *F. prausnitzii*, an important commensal bacteria of the human gut microbiota, employs an extracellular electron shuttle of flavins and thiols to transfer electrons to oxygen (16). In the study conducted by Okamoto *et al.*, contrary to our findings, *S. oneidensis*-produced flavins could be more effective in facilitating electron transfer than externally supplied flavins (21).

The body electron shuttles involve an electron transfer process that relies on the electron transfer between enzymes and cofactors. Several electron transfer processes are altered in cancer cells and modulated tumor metastasis and invasion (12). Accordingly, electrical medicine has been the focus of some studies for the treatment of cancer. “Optune” a new therapeutic device based on an electric circuit, was used as adjuvant therapy in patients with glioblastoma after chemoradiation therapy (22).

Previous studies demonstrated that metastatic human breast cells can be distinguished from non-transformed breast cells by redox reactivity (23). Here, we demonstrated that unlike different counts of rest-PBMCs, PMA-activated PBMCs, and tumor cells changed methylene blue color significantly, indicating different metabolism shunts and redox potential in these types of cells.

Recently, endogenous bioelectric signaling in the context of the immune response has been investigated by Paré *et al*. (24). Based on this study, general depolarization of the spatial gradients of transmembrane potential (V_{mem}) in *Xenopus laevis* embryos by genetically ion channel misexpression or pharmacological methods increased resistance to infection. Alteration of ionic currents that result from the movement of charged ions affected the protection of the microorganisms from bacterial infection. Another cellular electric current is derived from electron transfer through the oxidation-reduction agents, which were the basis of this study. We proposed that the alteration of gut redox potential due to the manipulation of the gut microbiome affects immune responses to the tumor and the efficiency of PD1 blockade immunotherapy as well (Fig. 5).

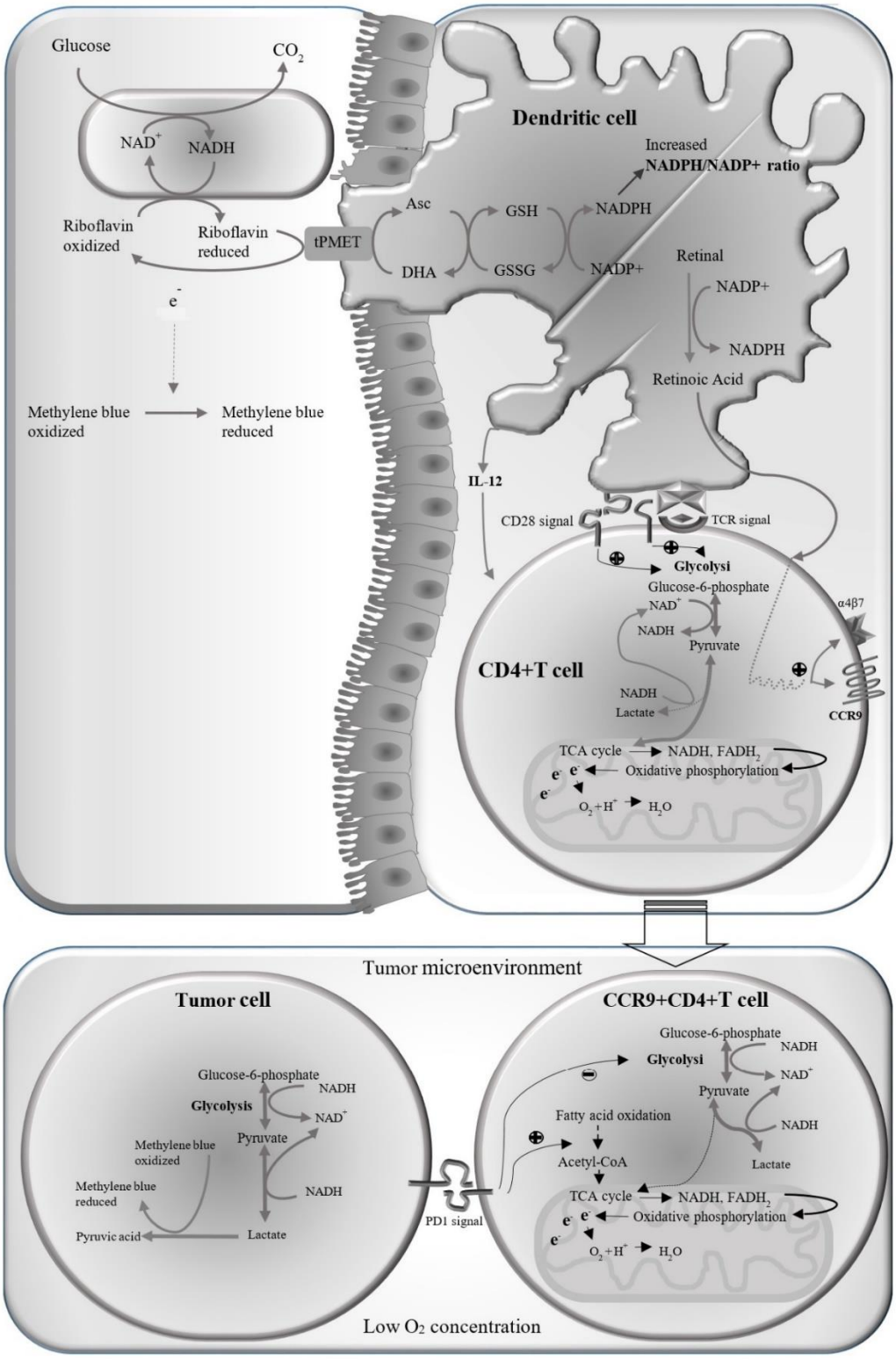

Fig. 5. Proposed mechanisms for cancer immunotherapeutic effect of gut electrogenic bacteria. Electrons transfer from the inner to the outer cell membrane by *Shewanella oneidensis* MR-1 reduced mucosal riboflavins. Electrons can enter mucus-monitoring DCs through the tPMET and eventually change the cellular ratio of redox mediators such as NADP/NADPH. Extracellular electron transfer-mediated redox potential can affect several metabolic pathways in immune and cancer cells including retinoic production by DCs, Warburg effect of cancer, and activated T cells in low O_2 concentrations in the tumor microenvironment and T cell receptor and PD1 signaling. tPMET, Trans plasma membrane electron transfer systems; DC, dendritic cells.

This association may be due to the direct contact of submucosal dendritic cells with the intestinal microbiome. Thymic stromal lymphopoietin and other factors induce dendritic cells (DCs) in gut-associated lymphoid tissues to express retinaldehyde dehydrogenase, which converts dietary vitamin A into retinoic acid. DC-produced retinoic acids induce T cells in the immunological synapse to express CCR9 (25). Routy *et al.* (9) studied the efficacy of oral supplementation with *Akkermansia muciniphila* and fecal microbiota transplantation in restoring response to anti-PD1 immune checkpoint therapy in mouse models of tumors. The authors suggested that this therapeutic effect was related to IL-12 by increasing the recruitment of $CCR9^{+}CXCR3^{+}CD4^{+}$ T lymphocytes into the tumor microenvironment. Hence, the expression of the IL-12 gene was assayed in the RNA extracted from the colon of the treated and control mice. According to the results, no significant change in the IL-12 gene expression between the control and other groups of study was observed.

Trans plasma membrane electron transfer systems transfer extracellular electrons into the cellular oxidation-reduction shunts, which consist of glutathione and ascorbate (12). An $NADP^{+}$ is reduced into NADPH through the retinaldehyde dehydrogenase activity. We proposed that the electron transfer from the *S. oneidensis* to DCs may change the NADPH/$NADP^{+}$ ratio, which can eventually lead to a change in retinoic acid production. The small number of mice in the study groups was the weakness of this study. The gut microbiome has been shown to have indirect effects on various aspects of human health. To further explore this hypothesis, it is recommended that future studies consider investigating a larger volume of samples.

CONCLUSION

Recent research has shed light on the potential therapeutic effects of electrogenic bacteria in cancer patients. The combined administration of *S. oneidensis* and anti-PD1 antibodies represents better tumor remission. While this study has provided valuable insights for immunotherapy using microbiomes and ICTs, further investigations in germ-free animals are necessary to conclusively determine the benefits of these bacteria in immunotherapy of patients with cancer.

Acknowledgments

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Conflict of interest statement

The authors declared no conflicts of interest in this study.

Authors' contributions

M. Khedri conceived the study, performed the experiments, analyzed the data, and drafted the manuscript. M. Nejati performed the experiments and edited the paper. M. Soheili and M. Salami revised the paper and provided useful comments. The finalized article was read and approved by all authors.

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