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Modulatory Effects of Metformin Alone and in Combination with Cimetidine and Ibuprofen on T Cell-related Parameters in a Breast Cancer Model

Fereshteh Taghipour[1,2], Omolbanin Oladpour[1,2], Mohammad Taghi Rezayati[2], Hossein Khorramdelazad[2], Maryam Nemati[3], Zahra Taghipour[4], Javad Masoumi[2], Zuhair Mohammad Hassan[5], and Abdollah Jafarzadeh[2,6,7]

[1] *Department of Immunology, School of Medicine, Rafsanjan University of Medical Sciences, Rafsanjan, Iran*
[2] *Molecular Medicine Research Center, Research Institute of Basic Medical Sciences, Rafsanjan University of Medical Sciences, Rafsanjan, Iran*
[3] *Department of Hematology and Laboratory Sciences, School of Para-Medicine, Kerman University of Medical Sciences, Kerman, Iran*
[4] *Department of Histology, School of Medicine, Rafsanjan University of Medical Sciences, Rafsanjan, Iran*
[5] *Department of Immunology, School of Medicine, Tarbiat Modarres University, Tehran, Iran*
[6] *Immunology of Infectious Diseases Research Center, Research Institute of Basic Medical Sciences, Rafsanjan University of Medical Sciences, Rafsanjan, Iran*
[7] *Department of Immunology, School of Medicine, Kerman University of Medical Sciences, Kerman, Iran*

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ABSTRACT

Metformin, cimetidine, and ibuprofen separately exhibit immunomodulatory and anti-tumorigenic effects. Herein, the impacts of metformin alone and in combination with cimetidine/ibuprofen on some Th1- and regulatory T (Treg) cell-related parameters were evaluated using a breast cancer (BC) model.

For establishing the BC model, four groups of Balb/c mice were challenged with the carcinoma cell line. After 11-30 days post-induction, they were treated intraperitoneally (with metformin (200 mg/kg), "metformin plus cimetidine (20 mg/kg)"; "metformin plus ibuprofen (20 mg/kg)", or with all three drugs in mentioned doses. Untreated BC and without tumor mice were enrolled as control groups. On day 31, splenic Th1 and Treg cell frequencies, serum interferon-gamma (IFN-γ), and transforming growth factor-beta (TGF-β) concentration, and intra-tumoral T-bet, TGF-β, and forkhead box protein P3 (FOXP3) expression were measured; using flow cytometry, enzyme-linked immunosorbent assay (ELISA), and real-time-PCR, respectively.

Treatment of the BC mice with metformin alone and in combination with cimetidine and/or ibuprofen enhanced the frequency of Th1 cells, and IFN-γ concentration, while it resulted in a decrease in the frequency of Treg cells, serum TGF-β concentration, and the expression of FOXP3 and TGF-β compared with un-treated BC mice. FOXP3 expression in the metformin-treated group was lower in mice who received combination therapy. Survival rate and body weight were increased, while tumor size and spleen index were reduced in mice treated with metformin

Corresponding Author: Abdollah Jafarzadeh, PhD;
Department of Immunology, School of Medicine, Rafsanjan University of Medical Sciences, Rafsanjan, Iran.
Tel: (+98 34) 3131 5220, Fax: (+98 34) 3428 0086, E-mail: Jafarzadeh14@yahoo.com

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alone and its combination with cimetidine and/or ibuprofen. No remarkable differences were found between metformin-treated mice and those who received combination therapies regarding Th1 and Treg cell percentages, TGF-β expression, body weight, tumor size, and spleen index.

The benefits of combinational therapy may be largely attributed to metformin. Immunotherapeutic potentials of metformin in cancers need further considerations.

Keywords: Breast neoplasms; Cimetidine; Ibuprofen; Metformin; Mice; T-lymphocytes

INTRODUCTION

Breast cancer (BC) is the second most prevalent cancer and the most frequent malignancy in women so that about 29.0% of newly diagnosed malignancies and about 14.0% of cancer-linked deaths in women were attributed to BC, worldwide.[1,2] The recognition and destruction of the malignant cells are one the most essential duties of the immune system, especially T cell-mediated arm immunity.[3,4] Among Th cells, the T-bet expressing Th1 cells secrete IFN-γ that promote the tumoricidal activity of natural killer (NK) cells, M1 macrophages, and $CD8^+$ cytotoxic T lymphocytes (CTLs).[4,5] Conversely, FOXP3 expressing Treg cells (that secrete IL-10, IL-35, and TGF-β) support tumor growth mainly via down-regulation of Th1 cell-related anti-tumor responses.[6,7]

Metformin (MET) is globally used for the treatment of type 2 diabetes mellitus.[8] The mitochondrion has been introduced as the main target of MET, where it suppresses the chain of electrons transport preventing various intracellular processes that need ATP.[8] Anti-tumoral effects of MET may be exerted through diminishing the glucose available to cancerous cells, suppressing the mTOR, eliciting the apoptosis via caspase 3 activation, preventing the cell cycle movement in G0/G1 phase via repressing P27, Cyclin D1, and Rb phosphorylation.[9] MET can suppress the signal transducer and activator of transcription-3 (STAT3) which is important for cancer cell survival.[10] MET decreases apoptosis of CTL cells and increases the expression of major histocompatibility complex (MHC) molecules.[11,12]

Cimetidine (CIM) can exert anti-tumor influences by preventing cancer cell proliferation, stimulating macrophages, up-regulating tumor suppressant cytokines, repressing angiogenesis via diminishing the VEGF expression inducing apoptosis, declining malignant cell adhesion, declining expression of E-selection and N-CAM, and stimulating proper immune responses.[13-15] The potent immunostimulatory properties were attributed to the CIM as an antagonist of the H2 receptor.[15]

Inflammatory cells can produce prostaglandins such as PGE2 via cyclooxygenase (COX)-mediated pathways, especially COX-2 activation.[16] Prostaglandins can directly stimulate cell expansion or indirectly induce cytokines such as IL-6 thus serving as tumor enhancing factors.[17] As a member of the non-steroidal anti-inflammatory drugs (NSAIDs), ibuprofen (IBU) represses inflammatory reactions via inhibiting COX-1 and COX-2 enzymes.[18] Moreover, IBU can exert anti-tumorigenic impacts through the induction of proper immune responses and histone modification.[19,20]

As mentioned above, MET, CIM, and IBU separately show anti-tumor effects. Therefore, we examine the immunomodulatory effects of MET alone and in combination with CIM and/or IBU in a model of BC in an attempt to clear their possible synergic impacts. Since T cells are the main effectors of anti-tumor immunity, here, the impacts of combination therapy using MET plus CIM and/or IBU on some Th1- (splenic cell frequency, serum IFN-γ levels, intra-tumoral expression of T-bet) and Treg (splenic cell frequency, serum TGF-β, intra-tumoral expression of FOXP3)cell-related parameters were investigated using a mouse BC model.

MATERIALS AND METHODS

Animals and Tumor Induction

A total of 84 female Balb/c mice with age 5-6 weeks were purchased from Iran's Pasteur Institute (Tehran, Iran) and housed in a normal condition (21±2°C temperature, 12 hours dark/light cycle and complete ventilation) with free access to water and pellet mouse food. The investigative procedure was also approved by an ethics committee affiliated with the Rafsanjan University of Medical Sciences (with ethical code: IR.RUMS.REC.1395.54). For tumor

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induction, the 4T1 carcinoma cell line was obtained from Iran's Pasteur Institute and expanded in RPMI-1640 medium supplemented with 15.0% of fetal bovine serum (FBS) (Gibco, Germany). The 4T1 cells (7×10^5 cells in 200 μL of RPMI) were injected into the right-side flank of mice. The mice were monitored daily until a tumor nodule emerged.

Treatment Protocol

In the tumor-bearing mice, the tumor mass was emerged about 7-8 days after tumor induction. However, the treatment with MET plus CIM and/or IBU was initiated when the sizes of the tumors were reached 100 mm^3 (about 11 days following tumor induction). At this time, the tumor size can be considered as an intermediate stage.[21] Treatment was begun at this time because most of the patients are admitted in stages II and III. In other studies, which used the same tumor model, treatment was initiated when the tumor size was reached this level.[22,23]

The drugs were dissolved in PBS (containing 10.0% ethanol) and filtered with a 0.2-micron filter and injected intraperitoneal from days 11 to 30post tumor induction (daily, during 21 days).The grouping of mice was done as follows: The BC was established in Groups 1-4of mice and were treated with MET(200 mg/kg), "MET (200 mg/kg) +CIM(20 mg/kg)"; "MET (200 mg/kg) +IBU (20 mg/kg)"; or "MET" (200 mg/kg) +CIM (20 mg/kg) +IBU (20 mg/kg)", respectively. The drug doses were determined according to the previous studies.[24-26] Group 5 was subjected to tumor induction, received the vehicle, and was considered as an untreated group. Group 6 was considered as a healthy control group that received the same volume of the vehicle without tumor induction.

Each group consisted of 14 mice, 7 of which were sacrificed on day 31 after tumor induction, and their blood, spleens, and breast tumor tissues were collected for further analysis. Of each group, 7 mice were also maintained and their survival was assessed up to 240 days after tumor induction.

Assessment of Spleen Index and Tumor Size

Mice and related spleens were weighed using an electronic scale and the spleen index was measured using the following formula:[22, 27]

Spleen index = spleen weight (g)/body weight (g) ×100

The sizes of the tumors were measured every three days; using a caliper. The volume of the tumor (V; mm^3) was calculated using the formula: $V=0.5\times d^2\times D$, in which d and D were considered to be the shorter- and the longer diameter of tumor mass, respectively.[22,28]

Measurement of Th1- and Treg Cell Percentages in the Spleen

The splenic Th1- and Treg cell frequencies were calculated by counting 10,000 cells; using a BD FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA) and BD CellQuest PRO software.

After removal of the spleen at day 31 after tumor induction, a single-cell suspension of each spleen was prepared; using a cell strainer (100 μm). The RBCs were then eliminated using an RBC lysis reagent. The splenic cells were washed in RPMI 1640 (supplemented with 10% FBS) and their viability was assessed using trypan blue staining.

The mouse multicolor flow cytometry kits were used to measure the splenic Th1- and Treg cell frequencies (R&D Systems, Minneapolis, USA; and San Diego, CA 2321, BioLegend, USA, respectively). The used reagents for Th1 cell counting were conjugated antibodies, including anti-CD4-PE and anti-IFN-γ-fluorescein and related isotype controls. The used reagents for Treg cell counting were as follows: anti-CD4-perCp, anti-FOXP3-Alexa Flour 488, anti-CD25-PE, and related isotype controls. The kits also contained a permeabilization/wash reagent (saponin and sodium azide 0.05%) and a fixation/ permeabilization reagent (1% formaldehyde, saponin).

Based on the manufacturer's instructions, the splenic cell suspensions were washed in PBS and re-suspended in fixation/permeabilization buffer, and then incubated at 2–8 C for 30 min. The cells were then centrifuged and suspended in the permeabilization/ wash buffer. After that, 10 μL of each specific conjugate antibody or corresponding isotype control antibody was added to the cells. Following the incubation, the unbound antibodies were excluded via a washing stage. The cells were eventually suspended in PBS for flow cytometry analysis.

Measurement of the Serum IFN-γ and TGF-β Levels

Blood samples were taken by cardiac puncture on day 31 after tumor induction and the serum specimens were isolated and preserved at -20°C until analyses. The serum levels of IFN-γ and TGF-β were determined

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using mouse ELISA kits (Biolegend, CA, USA) according to the manufacturer's guidelines.

Extraction RNA, cDNA Synthesis and Real-time PCR

Total RNA was separated from tumor tissues using the Trizol reagent (Bionner, Korea).To remove any contaminating genomic DNA, the separated RNA was exposed to DNase I (Thermo Scientific, EU). The quantity and purity of the RNA were assessed by measurement of the optical density (OD) at 260 nm and calculation of the OD260/OD280 ratio using spectrophotometry, respectively.

RNA samples were converted into cDNA using random hexamer and oligo (dT) primers provided by the cDNA synthesis kit (Bionner, Korea). TheT-bet, FOXP3, and TGF-β expression were assessed by real-time-PCR in triplicate using a real-time PCR system (Applied Biosystems, USA) by mixing SYBR green master mix (Biofact, Korea) with 200 ng of synthesized cDNA and 2 µL of suitable primers (Table 1). The real-time PCR protocol was planned as40 consecutive cycles of 95°C for 30 sec and 60°C for 30 sec, and eventually 72°C for 30 sec. The GAPDH gene was used as a housekeeping gene and the $2^{-\Delta\Delta Ct}$ formula was used for estimation of the interest gene expression. The melting curve analysis and quantitative data assessment were performed using an Applied Biosystems software version 1.1.308.111 (USA).

Histopathological Studies

After the scarification of mice on day 31, their lungs were collected, fixed in 10.0% formalin, and embedded in paraffin. Then the lung tissues were sliced into 5-micron sections and hematoxylin and eosin (H&E) staining was done for histological examination. The stained slides were examined using light microscopy to observe the existence of metastatic and histopathologic alterations.

Statistical Analyses

The data were presented as mean ± SEM. Statistical analysis was done; using ANOVA, Student *t*, Mann-Whitney U, Kruskal-Wallis, and $\chi 2$ tests as appropriate, and the P values less than 0.05 were considered significant. Statistical analysis software (SPSS version 20, Chicago, IL, USA) was used to compare the variables.

RESULTS

Treatment of the BC-bearing Mice with MET alone or in Combination with CIM and/or IBU Enhanced Body Weight

The percentage of weight gain was lower in un-treated BC-bearing compared to healthy control mice (0.53±2.5 vs 11.98±1.07, $p<0.0001$). The percentage of weight gain in BC mice treated with MET (9.27±2.05), "MET+CIM" (6.18±2.03), "MET+IBU" (9.16±2.03), or "MET+CIM+IBU" (10.32±1.70) were enhanced in comparison with un-treated cancerous mice ($p<0.01$, $p<0.09$, $p<0.02$, and $p<0.005$, respectively). No significant differences were identified between the MET-treated BC group and those treated with MET plus CIM/IBU concerning the percentages of weight gain (Figure 1A). The differences in the percentage of weight gain between the MET-treated group and mice treated with "MET + CIM", "MET + IBU" or all three drugs were not significant (p=0.31, p=0.97, and p=0.70, respectively).

Table 1. The used primers for assessment of the mRNA expression of investigated genes

Genes	Primer sequences	PCR product size (bp)
T-bet	Forward primer: 5′-TTCCCATTCCTGTCCTTCAC-3′ Reverse primer: 5′-CCACATCCACAAACATCCTG-3′	57
FOXP3	Forward primer: 5′-GGCCCTTCTCCAGGACAGA-3′ Reverse primer: 5′-GGCATGGGCATCCACAGT-3′	61
TGF-β	Forward primer: 5′-GCAGTGGCTGAACCAAGGA-3′ Reverse primer: 5′-AGCAGTGAGCGCTGAATCG-3′	56
GAPDH	Forward primer: 5′-CATGGCCTTCCGTGTTCCTA-3′ Reverse primer: 5′-GCGGCACGTCAGATCCA-3′	55

Treatment of the BC-bearing Mice with MET alone or in Combination with CIM and/or IBU Enhanced the Survival Rates

Animal survival rates were assessed during 240 days following tumor induction. All untreated BC mice died <50 days after induction of the tumor. About day 75 after tumor induction, the survival rate in mice treated with MET (7/7), "MET + CIM" (6/7), "MET+IBU" (6/7), or "MET + CIM + IBU" (7/7) was remarkably greater than that found in untreated BC group (0/7; $p<0.0001$, $p<0.005$, $p<0.005$, and $p<0.0001$, respectively). About day 150 after 4T1 cell inoculation, the survival rate in BC mice treated with MET (7/7) was significantly greater than that observed in mice treated with "MET + IBU" (1/7) or "MET + CIM" (1/7) ($p<0.005$). About day 150 after tumor induction, the survival rates in BC mice treated with "MET + CIM + IBU" (5/7) was significantly greater than those observed in the untreated BC group, and mice treated with "MET + IBU" (1/7) or "MET + CIM" (1/7) ($p<0.03$, $p<0.05$, and $p<0.05$, respectively). Thus, the rate of survival was enhanced in mice treated with MET alone or in combination with CIM and/or IBU compared with control untreated mice (Figure 1B). All MET-treated BC mice survived until about day 200 after tumor induction. After that 5/7 of mice were survived until day 240 days after tumor cell injection (Figure 1B).

Treatment of the BC-bearing Mice with MET alone or in Combination with CIM and/or IBU Enhanced the Spleen Index

The indexes of spleen were 0.48±0.03 in healthy mice, 3.39±0.48 in un-treated BC mice, 1.29±0.39 in MET-treated group, 1.92±0.62 in "MET + CIM"-treated mice, 1.55±0.27 in "MET + IBU"-treated group and 1.40±0.27 in "MET + CIM + IBU"- treated mice. Un-treated BC mice exhibited a greater spleen index than healthy mice ($p<0.0001$) (Figure 1C).

Treatment of the BC mice with MET, "MET + CIM", "MET + IBU", and "MET + CIM + IBU" decreased the spleen index as compared with un-treated BC mice ($p<0.0001$, $p<0.04$, $p<0.001$, and $p<0.001$, respectively). The differences of spleen index between the MET-treated group with those treated with MET plus CIM and/or IBU were not significant (Figure 1C). The differences in the percentage of the spleen index between the MET-treated group and mice treated with "MET + CIM", "MET + IBU" or all three drugs were not significant ($p=0.44$, $p=0.58$, and $p=0.82$, respectively).

Treatment with MET alone or in Combination with CIM and/or IBU Reduced Tumor Size and Tumor Weight in the BC-bearing Mice

The sizes of the breast tumors were measured every three days after beginning the treatment program. Tumor sizes in cancerous mice treated with MET alone or in combination with CIM/IBU on days 16, 19, 22, 25, 28, and 31 following tumor induction were significantly lower compared to untreated BC mice ($p<0.01$, $p<0.001$, $p<0.001$, $p<0.0001$, $p<0.0001$, and $p<0.0001$, respectively) (Figure 2A). No significant differences were detected between the MET-treated group and BC mice treated with MET plus CIM/IBU regarding the tumor sizes at different time points following tumor cell injection (Figure 2A).

The tumor tissues were collected and weighed on day 31 following 4T1 cell inoculation. The tumor weights in BC mice treated with MET (0.27±0.09 gram), "MET + CIM" (0.47±0.16 gram), "MET + IBU" (0.38±0.09 gram) and "MET + CIM + IBU" (0.29±0.08 gram) were significantly smaller than in non-treated BC mice (1.42±0.28 gram; $p<0.001$, $p<0.008$, $p<0.002$ and $p<0.001$, respectively) (Figure 2B). No significant differences were found between the MET-treated group with those mice treated with MET plus CIM and/or IBU regarding the tumor weight (Figure 2B).

Treatment of BC Mice with MET alone or in Combination with CIM and/or IBU Enhanced the Percentage of Th1 Cells while Reducing the Percentage of Treg Cells

Flow cytometry histogram concerning the Th1 and Treg cells was indicated in Figure 3A. The frequencies of the Th1- and Treg cells of spleen were 7.37±1.06 and 6.66±1.30 in the healthy group, 2.10±0.37 and 10.77±1.00 in un-treated BC mice, 5.85±0.64 and 4.94±0.50 in MET-treated group, 6.27 0.52 and 4.19±1.70in "MET + CIM"-treated mice, 7.61±1.39and 3.94±1.29in "MET + IBU"-treated group, and 3.90±0.24 and 2.97±0.89 in "MET + CIM+IBU"-treated group, respectively (Figure 3B and 3C). The un-treated BC mice exhibited a lower frequency of the splenic Th1 cells and a higher frequency of the splenic Treg cells when compared to healthy mice ($p<0.001$

and $p<0.04$, respectively).The BC mice that treated with MET, "MET+ CIM", "MET+ IBU", and "MET+CIM+IBU" exhibited higher frequencies of the splenic Th1 cells compared with un-treated BC mice ($p<0.001$, $p<0.0001$, $p<0.01$, and $p<0.01$, respectively) (Figure 3B).The differences in the percentage of the Th1 cells between the MET-treated group and mice treated with "MET+CIM" and "MET + IBU" were not significant ($p=0.62$ and $p=0.27$, respectively). The BC mice that were treated with MET displayed higher frequencies of the Th1 cells compared with mice treated with all three drugs ($p<0.03$) (Figure 3B).

The frequencies of the splenic Treg cells were reduced in treated BC mice that treated with MET-, "MET+CIM"-, "MET+IBU"-, and "MET+ CIM + IBU" compared to un-treated BC mice ($p<0.002$, $p<0.03$, $p<0.004$ and $p<0.0001$, respectively) (Figure 3C).

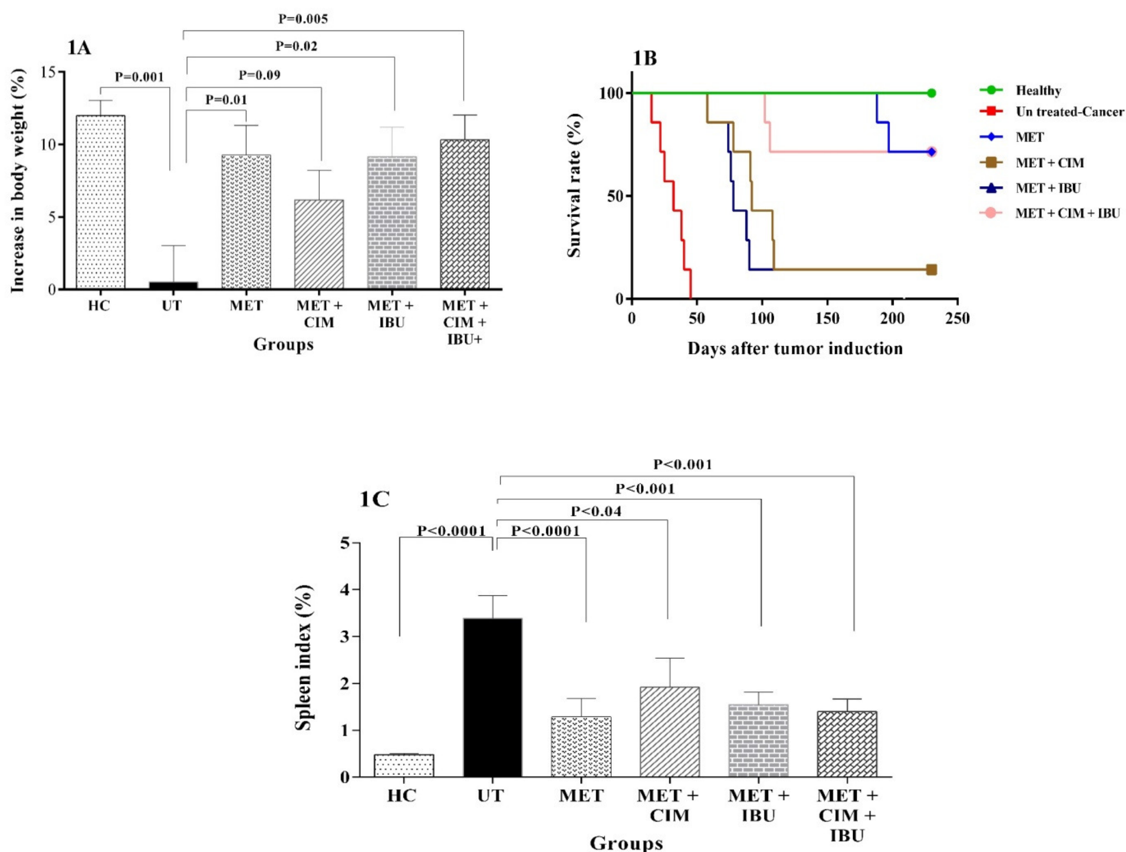

Figure 1. Treatment of the breast cancer (BC) mice with metformin (MET) alone or in combination with cimetidine (CIM) and/or ibuprofen (IBU) enhanced body weight and survival rate while reduced spleen index. Treatment with MET alone or in combination with CIM and/or IBU began daily, from days 11 to 30 post tumor induction. 1A: Percentage of weight gain in groups. The percentage of increase in body weight of mice was measured on day 31, before scarification. 1B: Survival rate in groups. Each group consisted of 14 mice, 7 of which were sacrificed on day 31 after tumor induction for immunologic analyses. Of each group, 7 mice were also maintained and their survival was assessed up to 240 days after tumor induction. Statistical analyses for comparison of the body the survival rate done using $\chi 2$ test. 1C: The spleen index in groups. The spleen index was measured on day 31post tumor induction, after scarification. Statistical analyses for comparison of body weight and spleen index were done using Student's t-test. HC: Healthy control mice; UT: Untreated BC mice; MET: Metformin-treated BC mice; "MET + CIM": "Metformin + Cimetidine"-treated BC mice; "MET + IBU": "Metformin + Ibuprofen"-treated BC mice; "MET + CIM + IBU": "Metformin + Cimetidine + Ibuprofen"-treated BC mice.

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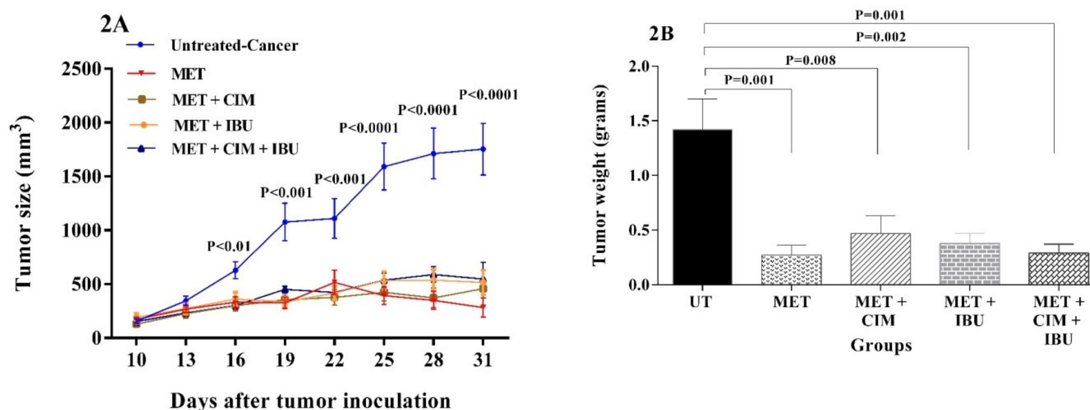

Figure 2. Treatment of the breast cancer (BC) mice with metformin (MET) alone or in combination with cimetidine (CIM) and/or ibuprofen (IBU) reduced tumor size and tumor weight. Treatment with MET alone or in combination with CIM and/or IBU began daily, from days 11 to 30 post tumor induction. 2A: Tumor size in groups. The sizes of the tumors were assessed every three days (on days 10, 13, 16, 19, 22, 25, 28, and 31 after tumor induction. The p values in each time point indicate the results of statistical analyses using the ANOVA test. 2B: Tumor weight in groups. The tumor weight of BC mice was measured on day 31post tumor induction, after scarification. Statistical analyses for the comparison of the tumor weight were done using Student's t-test. HC: Healthy control mice; UT: Untreated BC mice; MET: Metformin-treated BC mice; "MET + CIM": "Metformin + Cimetidine"-treated BC mice; "MET + IBU": "Metformin + Ibuprofen"-treated BC mice; "MET + CIM + IBU": "Metformin + Cimetidine + Ibuprofen"-treated BC mice.

The differences in the percentages of the splenic Treg cells between the MET-treated groups with those treated with MET plus CIM and/or IBU were not significant. The "MET + CIM + IBU"-treated BC mice exhibited lower splenic TH1 cells compared to MET-treated BC mice ($p<0.03$) (Figure 3B). The differences in the percentage of the Treg cells between the MET-treated group and mice treated with "MET + CIM", "MET + IBU" or all three drugs were not significant ($p=0.71$, $p=0.51$, and $p=0.14$, respectively).

Treatment of BC Mice with MET alone or in Combination with CIM and/or IBU Raised the IFN-γ Levels while Decreased the TGF-β Levels

The serum IFN-γ and TGF-β concentrations were 118.41±16.51 Pg/mL and 173.22±12.15 Pg/mL in healthy mice, 58.76±12.73 Pg/mL and 358.51±40.59 Pg/mL in un-treated BC mice, 122.38±15.00 Pg/mL and 128.74±22.29 Pg/mL in MET-treated group, 241.79±55.00 Pg/mL and 125.15±9.40 Pg/mL in "MET+ CIM"-treated mice, 177.96±30.77 Pg/mL and 192.69±33.62 Pg/mL in "MET + IBU"-treated group, and 232.67±72.03 Pg/mL and 89.84±18.76 Pg/mL in "MET + CIM + IBU"-treated group, respectively (Figure 4A and Figure 4B). The un-treated BC mice displayed lower serum IFN-γ concentrations and higher serum TGF-β concentrations in comparison with healthy mice ($p<0.01$ and $p<0.005$, respectively). The BC mice that treated with MET, "MET + CIM", "MET + IBU", and "MET + CIM + IBU" exhibited higher IFN-γ concentrations compared with un-treated cancerous mice ($p<0.008$, $p<0.01$, $p<0.01$, and $p<0.04$, respectively) (Figure 4A).The IFN-γ concentrations in "MET + CIM"-treated group was also higher than that observed in the MET-treated mice ($p<0.05$). The differences of the IFN-γ concentrations between the MET-treated group and mice treated with "MET + IBU" or all three drugs were not significant ($p=0.14$ and $p=0.17$, respectively) (Figure 4A).

The TGF-β concentrations in BC mice that treated with MET-, "MET + CIM"-, "MET + IBU"-, and "MET + CIM + IBU" were reduced compare to un-treated cancerous mice ($p<0.001$, $p<0.002$, $p<0.01$, and $P<0.001$, respectively) (Figure 4B). The differences of the TGF-β concentrations between MET-treated group and mice treated with "MET + CIM", "MET + IBU" or all three drugs were not significant ($p=0.88$, $p=0.14$, and $p=0.21$, respectively) (Figure 4B).

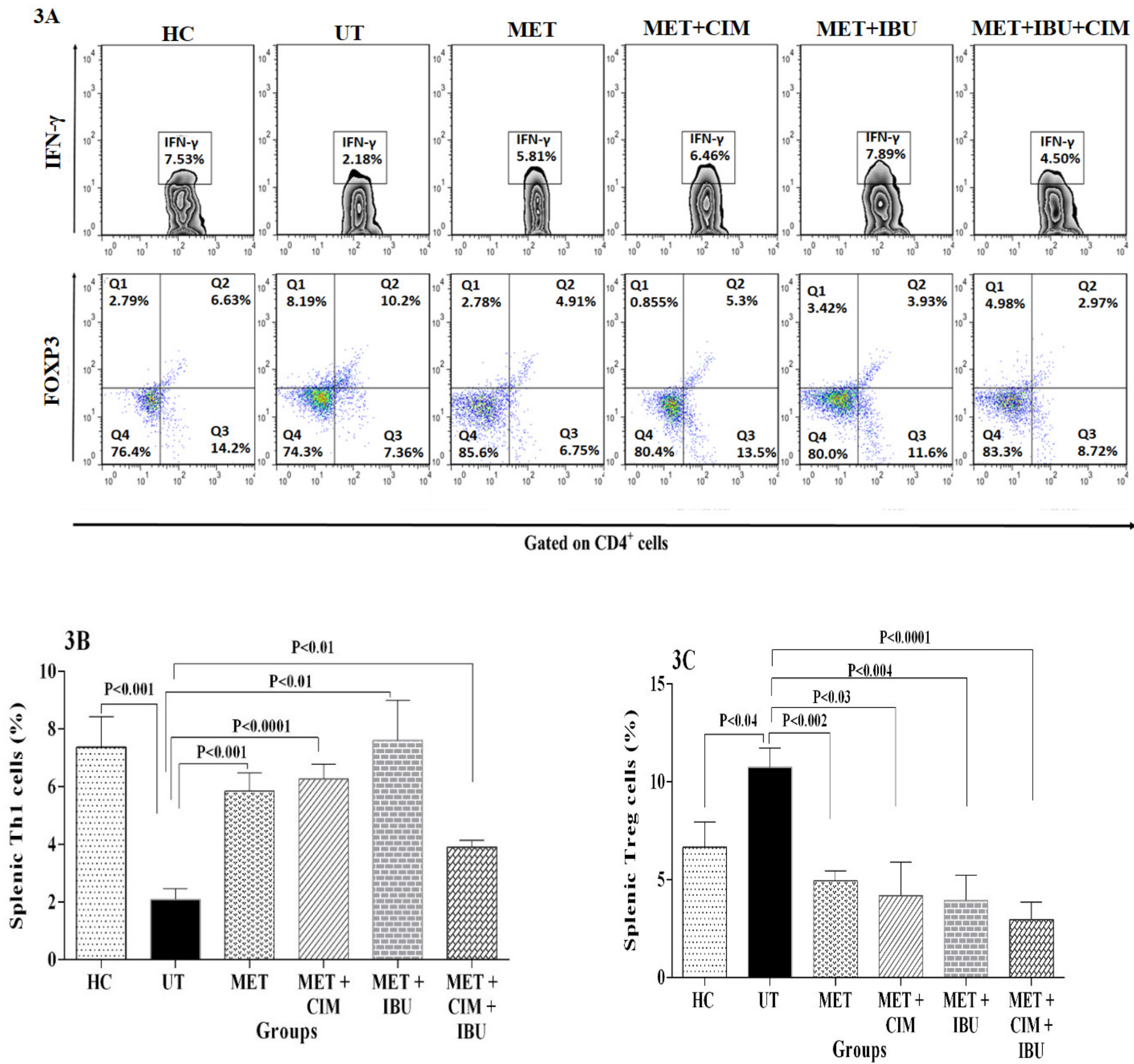

Figure 3. Treatment of the breast cancer (BC) mice with metformin (MET) alone or in combination with cimetidine (CIM) and/or ibuprofen (IBU) enhanced the percentage of the splenic Th1 cells, while reduced the percentage of the splenic Treg cells in BC-bearing mice.3A: Flow cytometry histograms: the splenic Th1 cells (in top) and Treg cells (in the bottom). 3B: The percentage of the Th1 cells in groups. 3C: The percentage of the Treg cells in groups. Treatment with MET alone or in combination with CIM and/or IBU began daily, from days 11 to 30 post tumor induction. The mice were sacrificed on day 31 after tumor induction. After removal of the spleen, the percentages of the splenic Th1- and Treg cells were determined using the flow cytometry method. Statistical analyses for the comparison of the percentage of the splenic Th1 and Treg cells were done using Student's t-test. HC: Healthy control mice; UT: Untreated BC mice; MET: Metformin-treated BC mice; "MET + CIM": "Metformin + Cimetidine"-treated BC mice; "MET + IBU": "Metformin + Ibuprofen"-treated BC mice; "MET + CIM + IBU": "Metformin + Cimetidine + Ibuprofen"-treated BC mice.

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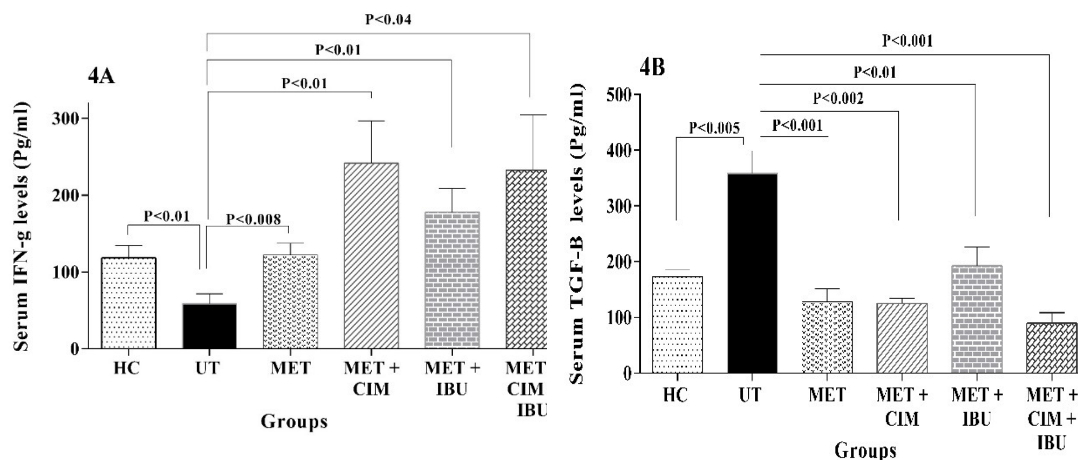

Figure 4. Treatment of the breast cancer (BC) mice with metformin (MET) alone or in combination with cimetidine (CIM) and/or ibuprofen (IBU)enhanced the serum levels of IFN-γ while reduced the serum levels of TGF-β. 4A: Levels of IFN-γ in groups. 4B: Levels of TGF-β in groups. Treatment with MET alone or in combination with CIM and/or IBU began daily, from days 11 to 30 post tumor induction. Blood samples were taken by cardiac puncture on day 31 after tumor induction. The serum levels of IFN-γ and TGF-β were determined; using the ELISA method. Statistical analyses for these experiments were done using Student's t-test. HC: Healthy control mice; UT: Untreated BC mice; MET: Metformin-treated BC mice; "MET + CIM": "Metformin + Cimetidine"-treated BC mice; "MET + IBU": "Metformin + Ibuprofen"-treated BC mice; "MET + CIM + IBU": "Metformin + Cimetidine + Ibuprofen"-treated BC mice.

Treatment of BC Mice with MET or "MET Plus CIM" Enhanced the Intra-tumoral Expression of T-bet

The fold changes expression of T-bet was 1.00±0.55 in un-treated BC mice, 3.17±1.13in MET-treated group, 5.20±1.30in "MET + CIM"-treated mice, 0.67±0.17 in "MET + IBU"-treated group, and 1.38±0.41 in "MET + CIM + IBU"-treated BC mice-treated group. Treatment of BC mice with MET or "MET + CIM" enhanced the T-bet expression as compared with un-treated BC mice (p<0.009 and p<0.0001, respectively). The T-bet expression in BC mice that were treated with MET, "MET + CIM" and "MET + CIM + IBU" was greater than that observed in the "MET + IBU"-treated group (p<0.03, p<0.01 and p<0.05, respectively). The T-bet expression in the "MET + CIM"-treated group was also higher than that observed in MET-treated mice (p<0.05). However, MET-treated mice expressed higher amounts of intra-tumoral expression of T-bet compared to the "MET + CIM + IBU"-treated BC group (p<0.05) (Figure 5A).

Treatment of BC mice with MET alone or in combination with CIM and/or IBU decreased the intra-tumoral expression of FOXP3 and TGF-β

The fold changes expression of FOXP3 and TGF-β were 1.00±0.33 and 1.00±0.20 in un-treated BC mice, 0.10 ± 0.04 and 0.49 ± 0.11 in MET-treated group, 0.42±0.19 and 0.36 ± 0.06 in "MET + CIM"-treated mice, 0.40±0.12 and 0.51±0.07 in "MET+IBU"-treated group, and 0.25±0.10 and 0.55±0.04 in "MET+CIM+IBU"-treated BC mice, respectively.

Treatment of BC mice with "MET", "MET+ CIM", "MET + IBU" and "MET + CIM + IBU" reduced the FOXP3 and TGF-β expression as compared with un-treated BC mice (p<0.003, p<0.02, p<0.005 and p<0.03 for comparison of FOXP3; p<0.02, p<0.003, p<0.02 and p<0.01for comparison of TGF-β, respectively) (Figure 5B and Figure 5C). The amount of the FOXP3 expression in the MET-treated group was lower than those measured in mice treated "MET+CIM", "MET + IBU" and "MET+CIM+IBU" (p<0.03, p<0.03, and p<0.05, respectively). (Figure 5B). The differences in the amount of TGF-β

expression between the MET-treated group and mice treated with "MET + CIM", "MET + IBU" or all three drugs were not significant (p=0.32, p=0.88 and p=0.62, respectively) (Figure 5C).

Treatment with MET alone or in Combination with CIM and/or IBU Reduced Lung Histopathological Changes in BC-bearing Mice

The alveolar spaces were reduced while thicknesses of alveoli walls are enhanced in untreated BC mice probably due to tumor cell metastasis. As indicated in Figure 6, the alveolar spaces are increased, while the thicknesses of alveoli walls and accumulated cells (likely metastatic colonies) were diminished in BC mice treated with MET alone and its combination with CIM and IBU compared to the untreated BC control group (Figure 6).

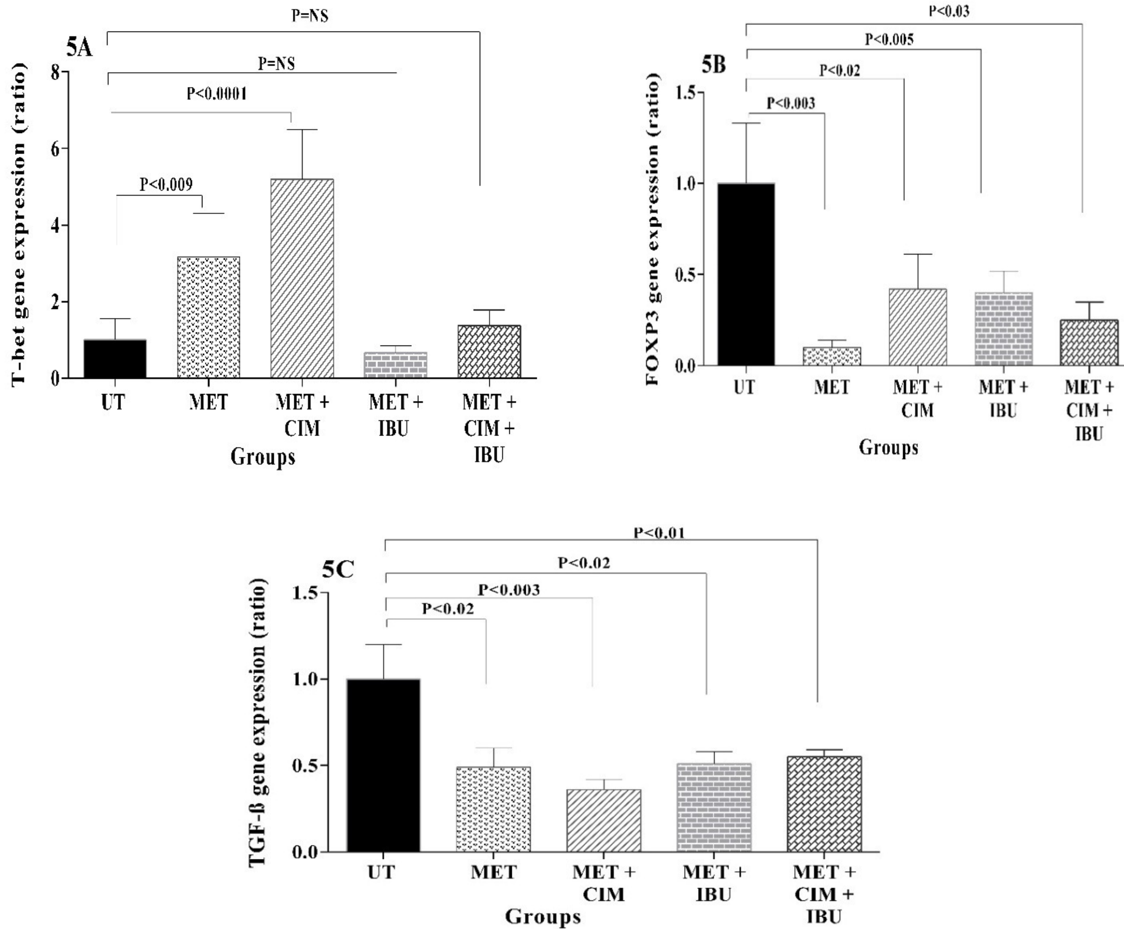

Figure 5. Treatment of the breast cancer (BC) mice with metformin (MET) or "MET plus cimetidine (CIM)" enhanced the T-bet expression, while treatment with MET alone or in combination with CIM and/or ibuprofen (IBU) reduced the expression of FOXP3 and TGF-β. 5A: The expression of T-bet in groups. 5B: The expression of FOXP3 in groups. 5C: The expression of TGF-β in groups. Treatment with MET alone or in combination with CIM and/or IBU began daily, from days 11 to 30 post tumor induction. The mice were sacrificed on day 31 after tumor induction. After removal of the tumor tissue, the intra-tumoral expression of the T-bet, FOXP3, and TGF-β was determined using the real time-PCR method. Statistical analyses for these experiments were done; using the Mann-Whitney U test or Student's t-test. HC: Healthy control mice; UT: Untreated BC mice; MET: Metformin-treated BC mice; "MET + CIM": "Metformin + Cimetidine"-treated BC mice; "MET + IBU": "Metformin + Ibuprofen"-treated BC mice; "MET + CIM + IBU": "Metformin + Cimetidine + Ibuprofen"-treated BC mice.

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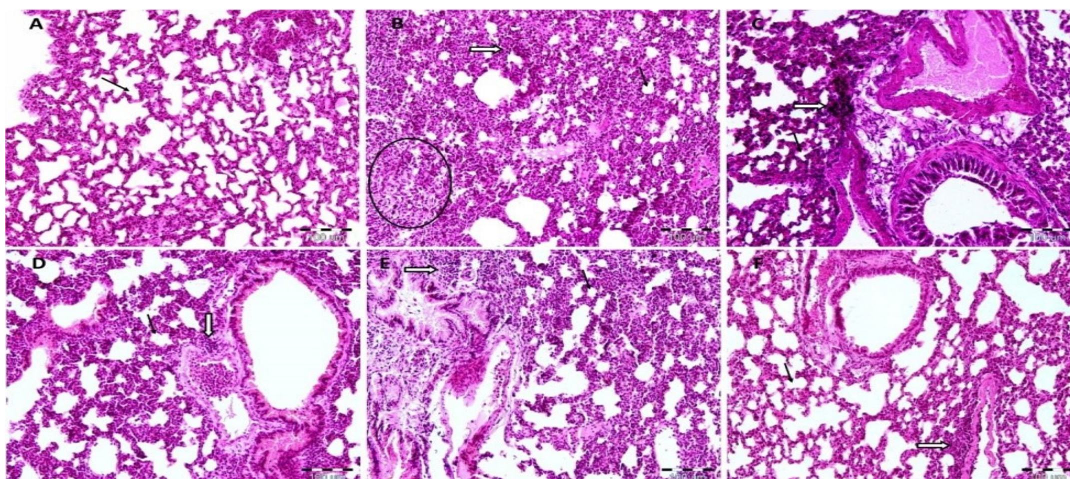

Figure 6. Treatment of the breast cancer (BC) mice with metformin (MET) alone or in combination with cimetidine (CIM) and/or ibuprofen (IBU) reduced lung histopathological changes. Treatment with MET alone or in combination with CIM and/or IBU began daily, from days 11 to 30 post tumor induction. The mice were sacrificed on day 31 after tumor induction. Histological examination on lungs was done using hematoxylin and eosin staining. 6A and 6B indicate the histological patterns of the lung in the healthy control- and untreated BC mice, respectively. 6C, 6D, 6E, and 6F indicate the lung patterns form MET, "MET + IBU", "MET + CIM" and "MET + CIM + IBU"-treated BC mice, respectively. The alveolar spaces were reduced while thicknesses of alveoli walls are enhanced in untreated BC mice probably due to tumor cell metastasis. As indicated the alveolar spaces are increased, while the thicknesses of the alveoli wall and accumulated cells (possibility metastatic tumor cells) were diminished in all treated mice compared to the untreated group.

DISCUSSION

We have observed that the percentage of weight gain and the survival rate was reduced, while spleen index was increased in untreated BC mice in comparison with the healthy group. Treatment of BC mice with MET alone or in combination with CIM/IBU increased the body weight and survival rate, while reduced the spleen index, tumor volume, and tumor weight. No significant differences were identified between MET-treated BC mice and those treated with MET plus CIM/IBU concerning the body weight, tumor volume, and tumor weight. About day 150 after 4T1 cell inoculation, the survival rate in BC mice treated with MET was remarkably greater than that in mice treated with "MET + IBU" or "MET + CIM". Thus, the beneficial effects of the used combinational therapeutic program can be largely attributed to MET. The anti-tumorigenic effects of MET can be exerted through several pathways such as mTOR suppression, protein synthesis inhibition, cell cycle stopping, induction of apoptosis, autophagy, decreasing blood insulin levels, stimulating the immune system, destruction of cancer stem cells, and angiogenesis suppression.[9]

The percentage of splenic Th1 cells and serum IFN-γ levels were also reduced, while the percentage of splenic Treg cells and serum TGF-β levels increased in untreated BC mice in comparison with the healthy group. Treatment of BC mice with MET alone or in combination with CIM and/or IBU enhanced the Th1 cell-related parameters (including their percentage and serum IFN-γ levels), while declined the Treg cell-related factors (including their percentage, serum TGF-β levels, and the intra-tumoral TGF-β and FOXP3 expression).

MET stimulates the $CD8^+$ tumor-infiltrating lymphocytes (TILs) to produce anti-tumor cytokines-TNF-α and IFN-γ- in mouse models of B16 melanoma and MC38 colon adenocarcinoma.[29] MET prevents $CD8^+$ TILs against apoptosis, thus enhances the number of $CD8^+$ TILs, and increases the efficacy of $CD8^+$ TILs to kill malignant cells.[11] MET prevents exhaustion of T cells and reinforces their capability to produce IL-2, TNF-α, and IFN-γ.[11] MET inhibits the tumor-promoting activity of myeloid-derived

suppressor cells (MDSCs), delays tumor progression, and elicitsTh1and CTL responses in a mouse model of colon cancer.[30] MET also reduces the frequency of FOXP3^{+}Treg cells in head and neck squamous cell carcinoma, increasing the ratio of CD8^{+}T/Treg cells.[31] MET-mediated suppression of Treg cells improves the anti-tumor activity of TILs to prevent tumor growth.[32]. Hypoxia and hypoxia-inducible factor-1a (HIF1α) promote the intra-tumoral Treg cell infiltration suppressing the CD8^{+} TIL activity.[33] MET mitigates hypoxia and deactivatesHIF1α causing Treg cell downregulation.[31,32] MET also downregulations PD-L1 thus promotes CD8^{+} T cell-induced cytotoxicity against malignant cells.[34,35]

The colorectal cancer patients with type 2 diabetes mellitus who were treated with MET exhibited a greater survival rate, higher CD8^{+} TILs, and lower CD163^{+} M2 macrophages in tumor-associated macrophages.[36] The patients with esophageal squamous cell carcinoma who were treated with metformin also displayed high numbers of CD8^{+}TILs,intra-tumoral of CD20^{+} B cells, and CD11c^{+} M1 macrophages, while the reduced number of CD163^{+} M2 macrophages and FOXP3 Treg cells.[37] Similarly, the treatment of BC patients with MET increased the number of CD4^{+} and CD8^{+} TILs and intra-tumoral expression of IFN-γ.[38]

CIM performs anti-tumorigenic effects through eliciting apoptosis in malignant cells, inhibiting VEGF expression, and suppression of angiogenesis.[13-15] CIM reduces tumor growth and improves the survival rate in an animal model of lung carcinoma.[39] CIM also promotes survival time in colorectal cancer patients.[40] Potent immunostimulatory properties were also attributed to CIM.[15] CIM enhances macrophages activation, increases NK cell activity, prevents the development of MDSC, inhibits the Treg cell functions, up-regulates anti-tumor cytokines, including IL-2, IL-12, IL-15, IFN-γ, TNF-α, and TNF-β, and improves the TIL activity.[15] The total number of CD3^{+} T cells, CD4^{+} T cells, and NK cells was enhanced in patients with gastrointestinal cancers who received CIM.[13,15,41]

IBU exerts anti-tumor effects via eliciting apoptosis in cancer cells, suppressing tumor cell expansion, triggering tumor suppressor protein, and preventing angiogenesis.[42,43] IBU potentiates anti-tumor immunity by stopping the PGE2 effects on the Th1- and Treg cells. IBU-treated mice with postpartum BC exhibit higher intra-tumoral numbers of T cells, more mature macrophages, and higher Th1/M1-derived cytokines such as TNF-α, IL-12, and IL-2.[19] Treatment with IBU prevents the loss of body weight, reduces tumor sizes, and promotes the survival rate in a mouse model of colorectal cancer.[44] IBU also reduces the risk of mortality in lung cancer patients.[45]

The aforementioned explanations indicate that MET, CIM, and IBU exhibit anti-tumoral activities. However, we have observed that the differences in the body weight, tumor size, spleen index, expression of TGF-β, percentages of Treg cells were not significant between BC mice treated with MET alone and those treated with MET in combination with MET plus CIM/IBU. Indeed, the BC mice treated with MET displayed higher frequencies of the Th1 cells compared with mice treated with all three drugs. The T-bet expression in MET-treated mice was also greater than that in the "MET + IBU"-treated mice and "MET + CIM + IBU"-treated group. Meanwhile, MET-treated BC mice exhibited a longer survival rate and lower FOXP3 expression.

BC mice-treated with "MET + CIM" also expressed higher serum IFN-γ levels and higher intra-tumoral T-bet expression compared with BC mice treated with MET, alone. Hence, the combination of "MET + CIM" may enhance the IFN-γ production and the T-bet expression in a synergic manner.

In summary, treatment of BC mice with MET alone or in combination promotes survival rate and percentage of weight gain, whereas declined tumor size, tumor weight, and spleen index. Mentioned treatment programs exert enhancing and reducing effects on the Th1 and Treg cell-related parameters, respectively. The number of Th1 cells, serum IFN-γ levels, and the expression of T-bet and FOXP3 are differentially exhibited between BC mice treated with MET, alone or its combination with CIM/IBU. The immunotherapeutic potentials of MET in cancers need more considerations.

CONFLICT OF INTEREST

There is no conflict of interest.

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