Immunological Assessment of Cryotherapy in Breast Cancer Patients

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Abstract. *Background: In murine studies, cryotherapy has induced antitumor immune responses associated with the rejection of tumors. However, the effects of freezing-induced immunomodulation in breast cancer (BC) patients remain unclear. Materials and Methods: Ten BC patients were prospectively divided into two groups: 1) cryotherapy followed by surgical excision and 2) surgical excision-alone. The cytokine profiles of plasma and peripheral blood mononuclear cells (PBMCs) were analyzed using flow cytometry following in vitro stimulation with the 30-mer MUC1 peptide. Results: No differences in the percentages of interferon-γ (IFN-γ)-producing cluster of differentiation (CD)4$^+$ or CD8$^+$ T cells and the plasma levels of IFN-γ, interleukin-1β (IL-1β), IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, tumor necrosis factor-α (TNF-α) and TNF-β were observed between these 2 groups, and PBMCs were not significantly altered. Conclusion: Alternations to the type 1 and 2 helper cytokine profiles were not detected in vitro in BC patients treated with cryotherapy-alone.*

Breast cancer (BC) is the second most common cancer in women. The prognosis for BC patients depends heavily on metastases and the average 5-year survival rate is approximately 25% (1). For patients with advanced BC, the treatment options are limited. Therefore, the development of new treatment modalities, such as cryotherapy, is of great importance for the prevention or treatment of BC patients.

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Cryoablation using extremely cold temperatures to destroy tumor tissues has been increasingly recognized as a highly efficient cancer therapy (2). Therefore, cryotherapy has been used in the clinic to treat several types of tumors, including breast, kidney, liver, esophagus, skin, prostate, lung and bone (2). Moreover, the efficiency of cryotherapy, combined with its limited side-effects, has been confirmed through pilot studies with early-stage breast cancer patients (3). Interestingly, cryotherapy induces both tumor-specific cytotoxic T-lymphocyte (CTL) and natural killer (NK) cell activities, which are associated with the rejection of tumor growth following tumor re-challenge in murine studies (4, 5). Therefore, freezing of tumors with cryoablation may trigger antitumor immunity *via* antigen-specific CTLs. However, cryotherapy-alone does not induce consistent immune responses and the adjunctive effects of immune stimulants lack clinical benefits (6).

BC cells express many types of tumor-associated antigens (TAAs), such as mucin 1 (MUC1), carcinoembryonic antigen (CEA) and epidermal growth factor receptor (EGFR)-2/neuregulin (HER-2/neu) (7). These TAAs have been used to induce antigen-specific CTLs (7). To induce efficient antigen-specific CTLs, antigenic peptides must be presented by major histocompatibility complex (MHC) class I and class II molecules on antigen-presenting cells (APCs). Dendritic cells (DCs) have been used in cancer vaccines as potent APCs (7). Following tumor cryoablation, DCs induce augmented tumor-specific CTL responses (8). Therefore, the activation of memory T-cells through immunotherapy and cryotherapy might augment CTL responses and prevent tumor recurrence (9). However, the effects of freezing-induced immune responses in BC patients remain unclear. Herein, we prospectively examined the effects of cryoablation therapy on immunomodulation in BC patients.

Materials and Methods

Study subjects. The Ethics Committee of the Jikei Institutional Review Board at Jikei University School of Medicine and the clinical study committee of Jikei University Kashiwa Hospital (No. 24-054 (6820)) reviewed and approved this study. All patients provided written informed consent and the procedures were performed in accordance with the Helsinki Declaration. The primary end-point of this study was the assessment of immune responses induced *via* cryotherapy and/or surgical excision.

Study design. We prospectively recruited 10 BC patients and randomly assigned these individuals to either cryotherapy followed by surgical excision (n=5) or surgical excision-alone (n=5) as a control. Plasma and PBMCs were collected prior to treatment and 3 weeks after cryotherapy. Twenty four days after cryotherapy, BC patients underwent surgical excision and plasma and PBMCs were collected again 4 weeks after surgical excision (Figure 1).

Cryotherapy. The cryoprobe was placed in 5 BC patients using an ultrasound (US) imaging system (AVIUS, Hitachi Medical Corp., Tokyo, Japan) (10, 11). Percutaneous cryotherapy was performed using a cryoablation system (CRYO-HIT, Galil Medical Ltd., Yokneam, Israel). High-pressure argon gas was used for freezing, producing a temperature of –185˚C at the tip of the needle probe. The tumor tissue was thawed using high-pressure helium gas, which induces heat at a temperature of 35˚C. Two freeze-thaw cycles were performed for cryosurgery. During cryotherapy, the formation of ice balls was monitored *via* a magnetic resonance (MR) imaging system (AIRIS II, Hitachi Medical Corp., Tokyo, Japan) (10, 11).

Immunohistochemistry. Formalin-fixed, paraffin-embedded human breast cancer tissue samples were stained with an anti-MUC1 antibody (Ma695; dilution 1:100; Novacostra Antibodies; Leica, Buffalo Grove, IL, USA), according to the manufacturers' directions (12, 13). When immunoreactive cells were detected in at least 5% of BC cells, samples were considered positive (13). Moreover, MUC1 staining was graded according to intensity (–: negative, +: positive or ++: strongly positive) (13).

Preparation of PBMCs for immunological analysis. PBMCs were obtained from BC patients before and after treatment. Within 24 h of blood collection, PBMCs were prepared using a Ficoll-Plaque Plus density gradient solution (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and stored at –80˚C in Bambanker freezing medium (Nippon Genetics Co., Ltd. Tokyo, Japan) without serum. After thawing, cell viability was confirmed as greater than 90% using a trypan blue exclusion assay.

Intracellular staining of interferon-γ (IFN-γ). PBMCs (1×10^6 cells/ml in each well) were cultured with a 30-mer MUC1 synthetic peptide, whose sequences corresponded to tandem repeats (10 μg/ml, TRPAPGSTAPPAHGVTSAPDTRPAPGSTAP) (Greiner Bio-One Co., Ltd, Tokyo, Japan) containing both MHC class I- and class II-restricted epitopes (14-16) in the presence of recombinant human (rh) interleukin -2 (IL-2) (10 U/ml, Shionogi, Osaka, Japan) and IL-7 (10 ng/ml, Peprotech, Rocky Hill, NJ, USA) for 9 days. The 30-mer MUC1 peptide-pulsed APCs induced cluster of differentiation (CD)4^+ and $CD8^+$ T-cell responses in an human leukocyte antigen (HLA)-unrestricted manner (14-16). PBMCs

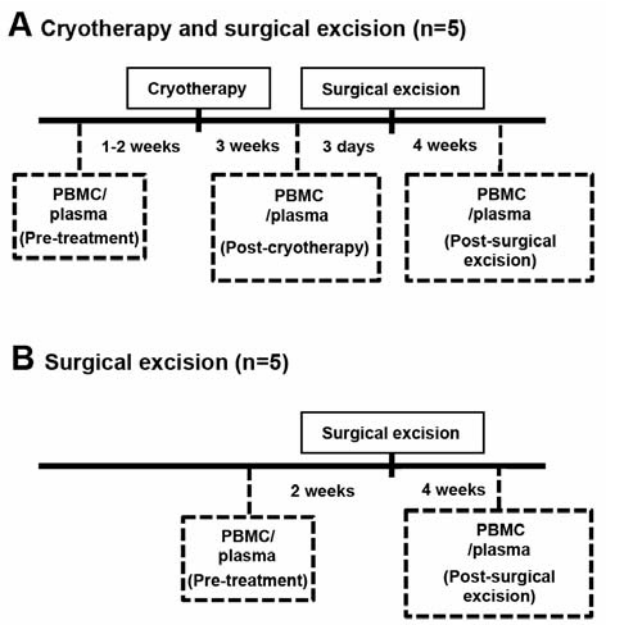

Figure 1. *Study profile. Ten breast cancer (BC) patients were prospectively divided into two groups: A) cryotherapy followed by surgical excision (n=5) and B) surgical excision-alone, as a control (n=5). Plasma and peripheral blood mononuclear cells (PBMCs) were collected before treatment and 3 weeks after cryotherapy. Twenty four days after cryotherapy, BC patients underwent surgical excision. Subsequently, plasma and PBMCs were collected again at 4 weeks after surgical excision.*

(1×10^5 cells/50 μl in each well) were re-stimulated with 10 μg/ml of 30-mer MUC1 peptides for 6 h using a GolgiPlug kit (BD Pharmingen, address), followed by staining with FITC-conjugated anti-human CD8, APC-Cy7-conjugated anti-human CD4 (eBiosciences, San Diego, CA, USA), and PE-conjugated anti-human IFN-γ or IL-10 monoclonal antibodies (mAbs) (BioLegend, San Diego, CA, USA). IFN-γ- or IL-10-producing $CD4^+$ or $CD8^+$ T-cells were analyzed using a MACSQuant analyzer (Miltenyi Biotec Inc., Auburn, CA, USA) and FlowJo analysis software (Tree Star, Inc., Ashland, OR, USA).

Cytokine expression in plasma or PBMCs. The secreted cytokine profile of PBMCs cultured with the MUC1 30-mer peptide was determined using the Human Th1/Th2 11plex Ready-to-Use FlowCytomix Kit, which allows for the measurement of human IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, tumor necrosis factor-α (TNF-α), and TNF-β in an immunoassay using a flow cytometer (eBiosciences). The cell culture supernatants and plasma collected from BC patients were stored at –80˚C. The cytokine concentrations were quantified according to the manufacturer's instructions. Individual cytokine concentrations were analyzed using FlowCytomix pro 3.0 software (eBiosciences).

Statistical analysis. The immunological parameters of the BC patients were evaluated using one-way analysis of variance. A *p*-value less than 0.05 was considered statistically significant.

Table I. *Patient characteristics*.

Patient no.	Age (years)	MUC1 status	Treatment	Pathology
1	32	++	Surgical excision	Ductal carcinoma
2	50	++	Surgical excision	Ductal carcinoma
3	60	++	Cryotherapy and surgical excison	Ductal carcinoma
4	43	+	Surgical excision	Ductal carcinoma
5	57	++	Cryotherapy and surgical excison	Ductal carcinoma
6	65	++	Cryotherapy and surgical excison	Ductal carcinoma
7	46	++	Cryotherapy and surgical excison	Ductal carcinoma
8	55	++	Cryotherapy and surgical excison	Ductal carcinoma
9	41	+	Surgical excision	Ductal carcinoma
10	39	++	Surgical excision	Ductal carcinoma

The intensity of MUC1 staining was graded according to the intensity (–; negative, +: positive, or ++: strongly positive).

Results

Patients' characteristics. Between August 2012 and May 2013, 10 BC patients who did not receive chemotherapy and hormone therapy were prospectively enrolled. The patients' characteristics are presented in Table I. Five patients received surgical excision-alone and the remaining 5 patients received cryotherapy, followed by surgical excision. MUC1 expression in the membrane or cytoplasm of BC cells was observed in all 10 BC patients (Table I). All 5 patients successfully underwent cryotherapy and surgical excision. The treatment of all 5 BC patients with cryotherapy resulted in elimination of tumor cells.

Assessment of MUC1-specific immune responses through cryotherapy. The MUC1 antigen is expressed in most BC tissues (91%) (17) and MUC1 expression was detected in all enrolled BC patients. Therefore, PBMCs from BC patients treated with cryotherapy and/or surgical excision were stimulated with 30-mer MUC1 peptides, including both MHC class I- and class II-restricted epitopes in an HLA-unrestricted manner (14-16) *in vitro* to determine whether this treatment could induce MUC1-specific $CD4^+$ and $CD8^+$ T-cell responses. Upon stimulation of PBMCs with the 30-mer MUC1 peptide, IFN-γ production was detected in both $CD4^+$ and $CD8^+$ T cells (Figure 2A). After cryotherapy and/or surgical excision, we did not detect alterations in IFN-γ-producing $CD4^+$ and $CD8^+$ T cells *in vitro* (Figure 2B and C). In addition, $CD4^+$ and $CD8^+$ T-cells secreted baseline levels of IL-10 before and after cryotherapy and/or surgical excision.

Cytokine production in PBMCs. To assess the cytokine profiles of T-helper 1 (Th1) and T-helper 2 (Th2) cells after cryotherapy and/or surgical excision, PBMCs were stimulated with the 30-mer MUC1 peptide *in vitro*. The culture supernatants were analyzed using the FlowCytomix kit. After cryotherapy-alone, no significant alternations to Th1 cytokines, such as IL-2, IFN-γ, IL-12p70 and TNF-β, and Th2 cytokines, such as IL-4, IL-5, IL-10 and IL-6, were observed before therapy (Figure 3). Moreover, there were no significant differences between cryotherapy and surgical excision in terms of cytokine secretion from PBMCs (Figure 3). In addition, IL-1β, IL-8 and TNF-α were secreted at baseline levels from BC PBMCs before and after treatment.

Cytokine levels in plasma. To assess the cytokine profiles of Th1/Th2 cells in BC patients after cryotherapy and/or surgical excision, the plasma from these patients was directly analyzed using FlowCytomix. The plasma levels of Th1 cytokines, such as IL-2, TNF-α and TNF-β, and Th2 cytokines, such as IL-6 and IL-10, were not altered after treatment compared with the levels observed before treatment (Figure 4). Moreover, there were no significant differences between the cryotherapy and surgical excision groups in terms of plasma cytokine levels (Figure 4). In addition, IFN-γ, IL-1β, IL-4, IL-5, IL-8 and IL-12p70 were secreted at baseline levels in the plasma before and after treatment.

Discussion

The cryoablation of tumors through repeated deep freezing and thawing reportedly induces tumor necrosis and apoptosis, thereby providing antigen sources to immature DCs for the induction of antigen-specific antitumor immunity (18, 19). Using cryotherapy in BC patients, we demonstrated that immunomodulation was not induced *in vitro*.

While distant tumor regression after cryotherapy has been reported in clinical trials, induction of immunomodulation through cryotherapy-alone has been controversial (6). In the present study, BC patients received cryotherapy, followed by surgical excision to assess the effects of this treatment. We observed the elimination of tumor cells in surgical samples from

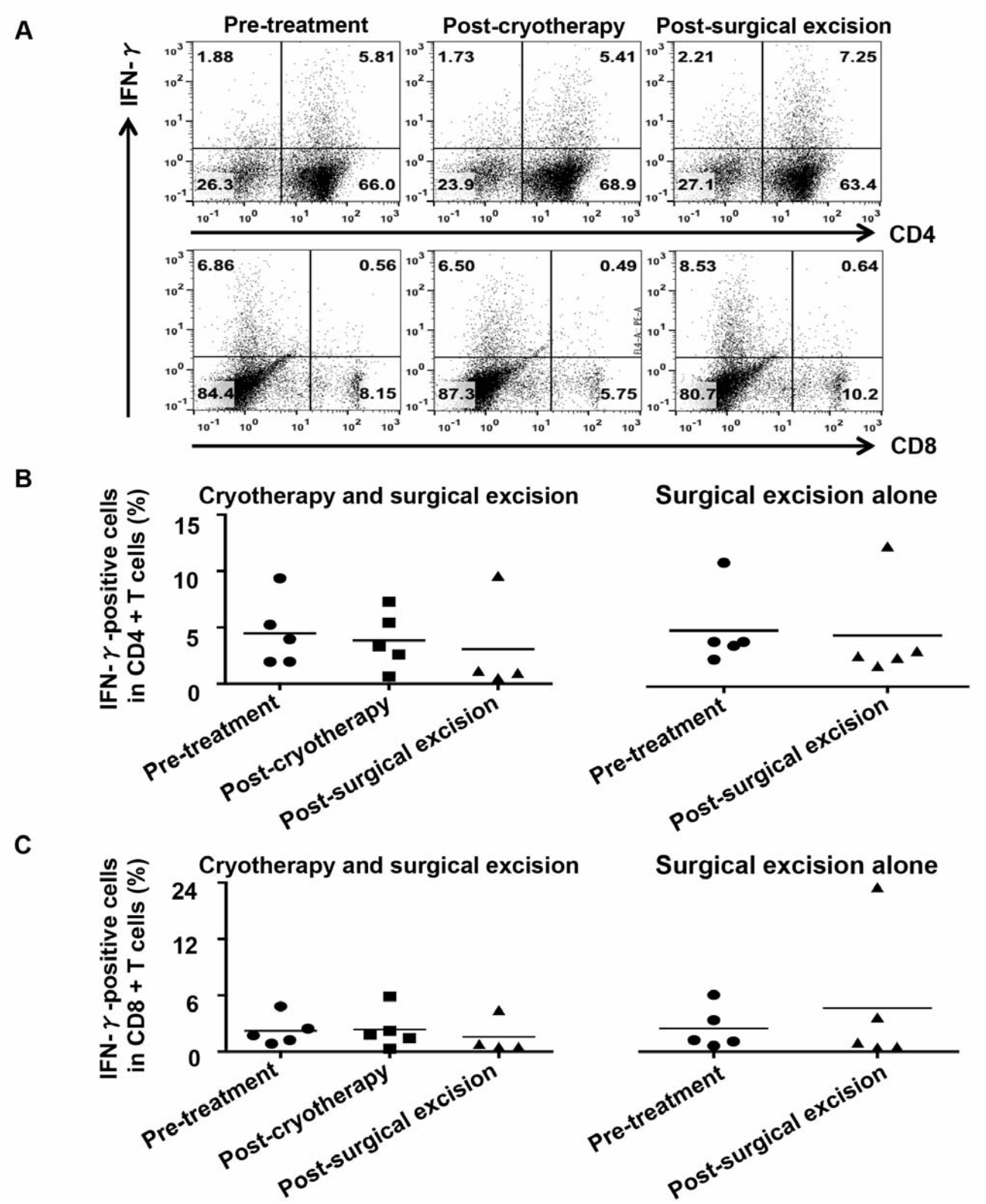

Figure 2. *Intracellular staining of IFN-γ. A. Dot blots of peripheral blood mononuclear cells (PBMCs) analyzed for IFN-γ-producing CD4+ and CD8+ T-cells upon stimulation with the 30-mer MUC1 peptide in vitro are shown. The percentages of IFN-γ-producing CD4+ (B) and CD8+ T-cells (C) from the 2 groups (cryotherapy followed by surgical excision; left panel and surgical excision alone; right panel) were assessed.*

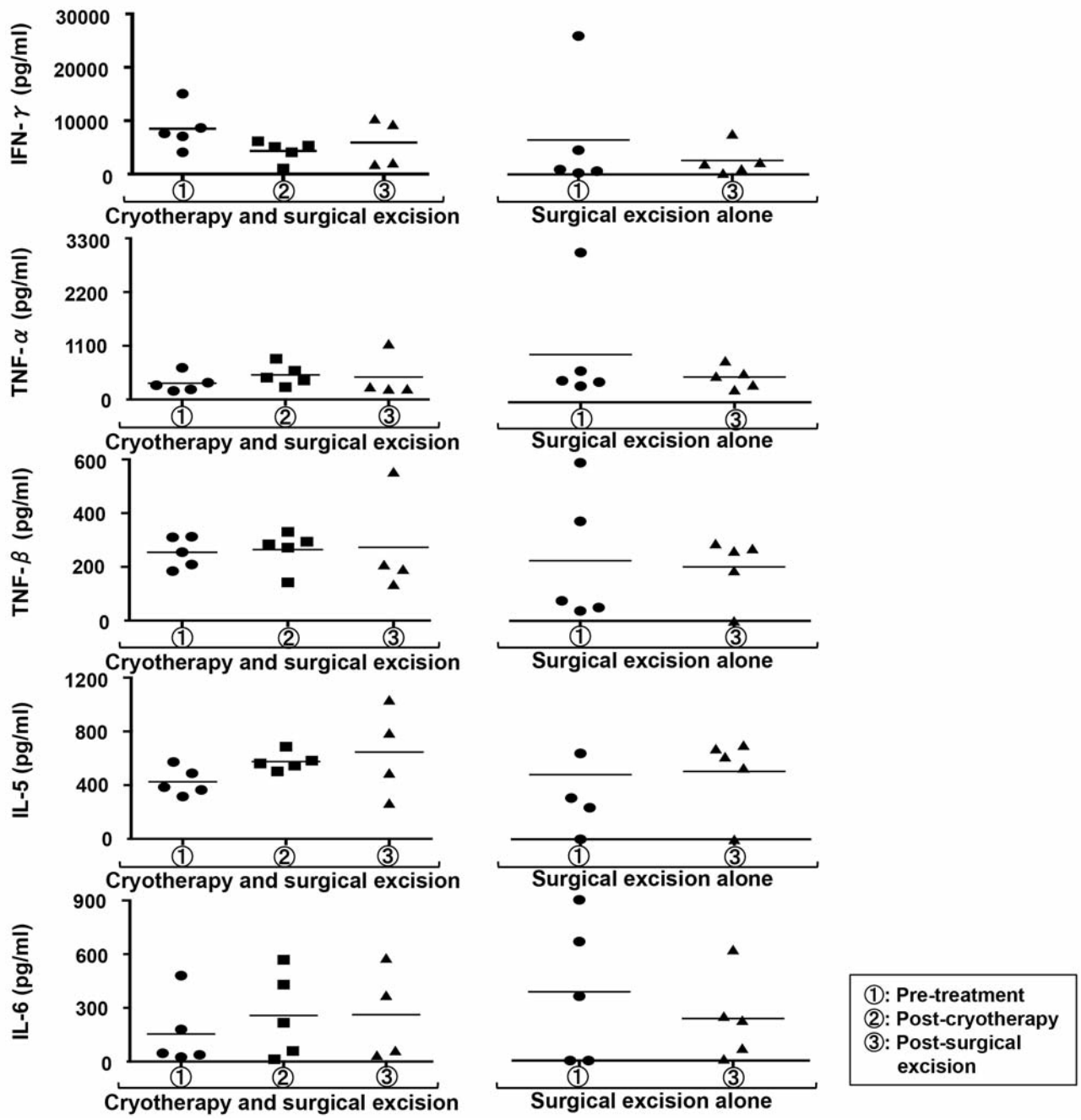

Figure 3. *Cytokine production in peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMCs) from breast cancer (BC) patients treated with cryotherapy and surgical excision (left panel) or surgical excision-alone (right panel) were stimulated with the 30-mer MUC1 peptide in vitro. The levels of IFN-γ, TNF-α, TNF-β, IL-5 and IL-6 secreted into the culture supernatants are shown.*

BC patients treated with cryotherapy. To assess whether cryotherapy-alone induces immune responses in BC patients *in vivo* and *in vitro*, we first analyzed IFN-γ-producing T-cells upon stimulation with the antigenic MUC1 peptide. MUC1 is a major tumor antigen overexpressed in human breast cancers (17) and comprises of a variable number of carbohydrates and 20-amino-acid repeat sequences, referred to as variable number tandem repeats (VNTRs). Indeed, the MUC1 antigen represents an appropriate target for breast cancer immunotherapy (20). We used a 30-mer MUC1 peptide (TRPAPGSTAPPAHGVTSAP

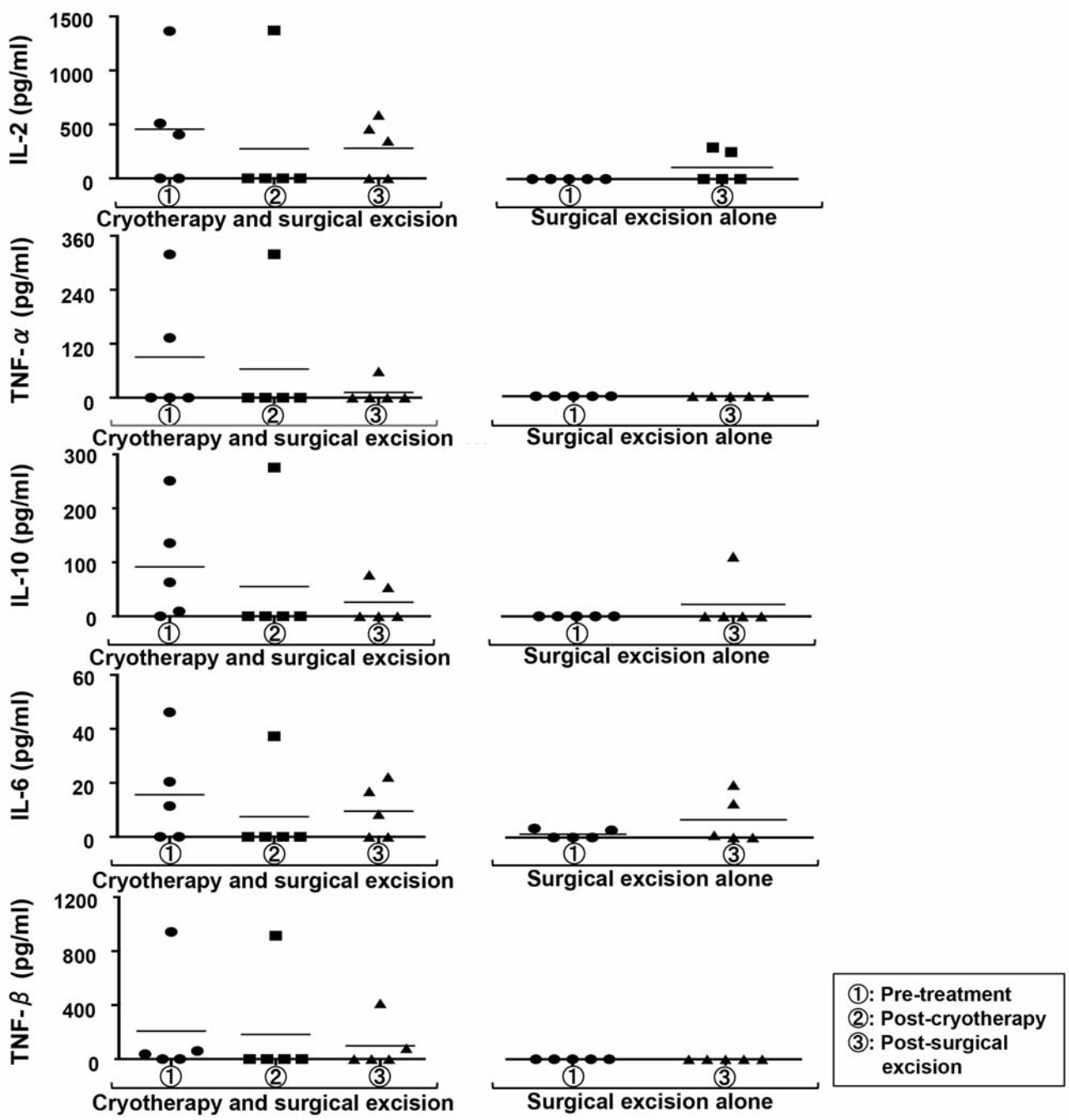

Figure 4. *Plasma cytokine levels. Plasma from breast cancer (BC) patients treated with cryotherapy and surgical excision (left panel) or surgical excision-alone (right panel) was analyzed. The plasma levels of IL-2, TNF-α, IL-10, IL-6 and TNF-β are shown.*

DTRPAPGSTAP), including SAPDTRPA, which is a dual epitope presented to both $CD4^+$ and $CD8^+$ T-cells in an HLA-unrestricted manner (14-16). Before therapy, MUC1-specific IFN-γ-producing $CD4^+$ and $CD8^+$ T-cells were detected after stimulation with the 30-mer MUC1 peptide *in vitro*, suggesting the presence of MUC1-specific CTLs in BC patients. No significant increases in MUC1-specific IFN-γ-producing $CD4^+$ and $CD8^+$ T-cells were observed after cryotherapy and/or surgical excision, suggesting that cryoablation and/or surgical excision is not enough to induce immunomodulation. In murine

studies, cryotherapy- alone was not enough to induce efficient antitumor immunity; however, the combination of cryotherapy and immunomodulating agents, including protein-bound polysaccharides such as polysaccharide-K (PSK), CpG oligodeoxynucleotides (CpG ODN) and TNF-α, synergistically augmented antitumor immune responses (18, 21, 22). Moreover, the combination of cryotherapy with irradiation or a low dose of chemotherapeutic agents, such as 5-fluorouracil (5-FU), is useful to control and eradicate cancer (23, 24). Therefore, cryotherapy might be used to induce immunomodulation in patients with advanced BC when combined with irradiation or chemotherapy, generating efficient clinical responses.

To further examine immunomodulation, it was important to analyze Th1/Th2 cytokine profiles. Three weeks after cryotherapy, PBMCs and plasma were collected from each BC patient and the BC patients subsequently received surgical excision. We did not detect immunomodulation in the plasma of BC patients. Moreover, alterations to the Th1/Th2 profiles in PBMC supernatants were not detected, even when these cells were stimulated with the 30-mer MUC1 peptide *in vitro*. Although tumor stage and technical factors influence immune responses, cryoablation-alone might not be sufficient to induce strong immunomodulation. Moreover, immunomodulation through cryotherapy might be transient and, thus, induction of long-term antitumor immune responses is required to prevent recurrence.

In conclusion, cryotherapy-alone in BC patients is not sufficient to cause detectable immunomodulation *in vitro*. However, a limitation of the study is the relatively small sample size of BC patients. Therefore, further studies are required to assess immunomodulation through cryotherapy using larger sample sizes.

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Conflicts of Interest

The Authors declare that they have no competing interests.

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