ORIGINAL RESEARCH

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Immunomodulatory effect of peritumorally administered interferon-beta on melanoma through tumor-associated macrophages

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An imbalance of immunosuppressive cells and cytotoxic cells plays an important role in the tumor-bearing host. Together with regulatory T cells (Tregs), tumor-associated macrophages (TAMs) play roles in maintaining the tumor microenvironment. Since interferon beta (IFN-β) has been clinically used for the treatment of malignant melanoma, we investigated the immunomodulatory effect of IFN-β during melanoma growth to elucidate the effects of IFN-β on the tumor microenvironment by using the B16F10 melanoma model. Peritumorally administered IFN-β significantly decreased the mRNA expression and production of Th2-related chemokines, which suppressed the recruitment of Tregs in B16F10 melanoma. Since the administration of IFN-β augments the expression of PD-1 on TILs, the co-administration of anti-PD-1 Ab augmented the therapeutic effect of IFN-β for the treatment of B16F10 melanoma. Moreover, in parallel with the mouse model, in the human system, IFN-β decreased the production of Th2-related chemokines and augmented the production of Th1-related chemokines from monocyte-derived M2 macrophages. Since these immunomodulatory effects of IFN-β on macrophages were also observed in the lesional skin of human in-transit melanoma, our present data suggest one of the possible immunomodulatory effects of IFN-β and support the possibility of IFN-β in combination with anti-PD-1 Ab for the treatment of melanoma.

Introduction

IFN-β inhibits the growth of melanoma cells and induces cross-priming of $CD8^+$ T cells by dendritic cells *in vitro* and *in vivo*,[1,2] and has been clinically used for the treatment of malignant melanoma.[3,4] In melanoma patients, peritumoral injection of IFN-β recruits effector cells including $CD8^+$ and TIA1-positive cytotoxic T cells (CTLs) into the tumor microenvironment,[5] which might explain one of the possible mechanisms for the therapeutic effects of IFN-β in the treatment of melanoma. Although there is supportive evidence for the use of type I interferon as an adjuvant therapy for malignant melanoma, tumor regression occurred in only 15–20% of patients treated with IFN-β, and significantly prolonged survival was not attained by IFN-β treatment.[6] This discrepancy could be explained by the expression of PD-L1 on human melanoma cells.[7]

The PD-1/PD-L1 pathway plays a critical role in the tumor immune response in human melanoma.[7,8] Hino et al. demonstrated that the expression of PD-L1 on tumor cells is an independent prognostic factor for human melanoma [7] that correlates with the vertical growth of primary tumors and determines the prognosis of melanoma patients.[7] Based on these findings, an anti-PD-1 Ab, nivolumab, was developed and used clinically in advanced melanoma patients. Nivolumab significantly prolonged survival in patients with metastatic melanoma, but only 31% of patients who received nivolumab monotherapy experienced objective tumor regression.[8] Therefore, optimal reagents that enhance the antitumor immune response by nivolumab are necessary to improve the treatment for melanoma.[9]

Another target for PD-1/ PD-L1 blockade therapy is TAMs. TAMs in murine and human melanoma express high levels of PD-L1, which promotes an immunosuppressive microenvi-

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ronment.[10-13] Together with Tregs, immunosuppressive TAMs promote an immunosuppressive environment in the tumor-bearing host.[10,12,15-17] Alternatively activated M2 macrophages represent the majority of TAMs, and the main population of inflammatory cells in solid tumors.[18-20] Notably, TAMs are a heterogeneous population of cells that could be polarized to M1-like macrophages in the tumor microenvironment.[21] These reports suggest that TAMs could be a target of immunotherapy for melanoma.

Results

Immunomodulatory effects of peritumorally injected IFN-β on the tumor microenvironment of B16F10 melanoma

To investigate the immunomodulatory effects of IFN-β on the tumor microenvironment *in vivo*, we used the mouse B16F10 melanoma model. First, we evaluated the subpopulation of tumor-infiltrating T cells and Th1/Th2-related chemokines from each group of tumors by qRT-PCR. The peritumoral administration of IFN−β significantly decreased the expression of Foxp3 and GATA3 and increased the expression of T-bet in the tumor microenvironment (**Fig. 1A**). There was no significant difference in the expression of RORγ. Moreover, the administration of IFN−β significantly decreased the mRNA expression of Th2 chemokines (CCL17, CCL22) and increased the mRNA expression of Th1 chemokines (CXCL9, CXCL11) (**Fig. 1B**) in the tumor microenvironment. The mRNA expression of CCL2 and CXCL10 was also augmented, although it was not statistically significant.

Next, to confirm that the increased mRNA expression leads to protein expression, we examined the immunomodulatory effects of IFN-β on the production of Th1/Th2 chemokines from TAMs in B16F10 melanoma. We isolated CD11b$^+$ cells from tumors, as described in the Materials and Methods. The CD11b$^+$ cells were cultured for 48 h with Lipopolysaccharides (LPS), and the culture supernatant was

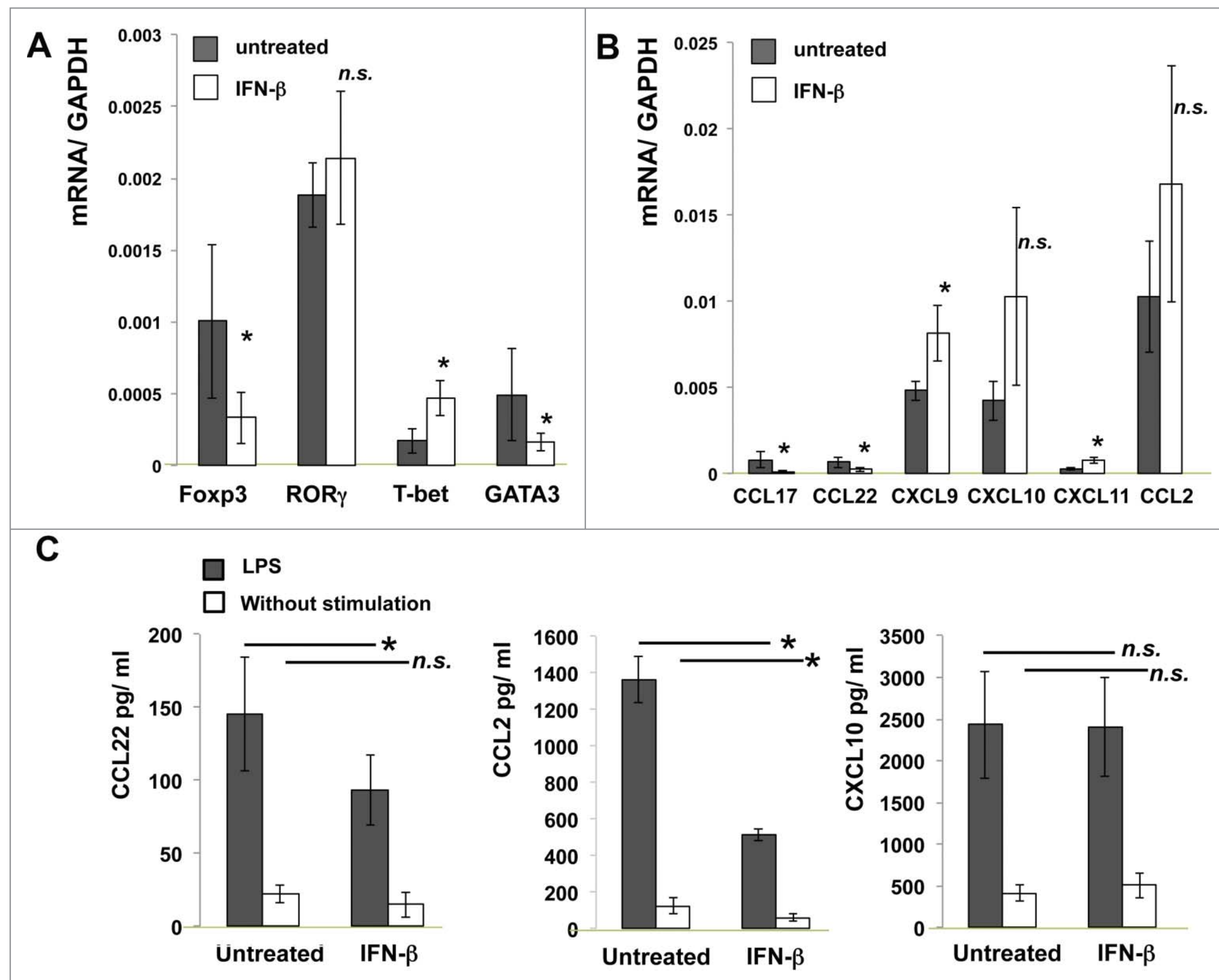

Figure 1. Peritumorally administered IFN-β modifies the chemokine production of TAMs in B16F10 melanoma. The expression of T-bet, GATA3, Foxp3, and RORγt mRNA (**A**) and chemokines (**B**) in B16F10 melanoma was analyzed by quantitative RT-PCR by using the ΔCt method. CD11b$^+$ cells isolated from tumors of indicated groups were stimulated with or without LPS (0.5 μg/mL), and cytokine production was measured by ELISA (**C**). One representative experiment of three is shown; error bars represent the standard deviation within triplicates. * marks a significant ($P < 0.05$) difference.

subsequently quantified by enzyme-linked immunosorbent assay (ELISA). Consistent with the mRNA expression, the production of CCL22 was significantly decreased, and there was no significant difference in the production of CXCL10 (**Fig. 1C**). In contrast to mRNA expression, the production of CCL2 was significantly decreased (**Fig. 1C**). Unexpectedly, CXCL9 and CXCL11 were not detected (data not shown).

Peritumorally injected IFN-β decreased the recruitment of Tregs and PD-L1-expressing tumor-associated macrophages (TAMs) and augmented the activated T cells in B16F10 melanoma

As described above, the administration of IFN-β decreased the production of CCL22, which is related to the recruitment of Tregs. We evaluated the ratio of $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs in the tumor by flow cytometry. Whole tumor cells were analyzed after stimulation with IFN−β. Consistent with the production of CCL22, the ratio of Foxp3 Tregs in $CD4^{+}$ T cells was significantly decreased (**Fig. 2A**). In addition, in parallel with the decreased production of CCL2, the PD-L1-expressing $CD11b^{+}$ TAMs were also decreased (**Fig. 2B**). Moreover, flow cytometry analysis revealed that the expression of CD69 on $CD8^{+}$ cells, but not $CD4^{+}$ cells, was significantly increased in B16F10 melanoma (**Fig. 2B**). The expression of PD-1 on $CD4^{+}$ cells and $CD8^{+}$ cells was also increased (**Fig. 2B**). Since we could not detect a significant difference in the production of Th1 chemokines, we examined the chemotactic activity of culture supernatant from $CD11b^{+}$ TAMs in each group. The culture supernatant from $CD11b^{+}$ TAMs significantly increased the chemotactic activity for TRP-2-specific $CD4^{+}$ T cells (**Fig. 2C**), which might suggest that chemotactic factors from $CD11b^{+}$ TAMs recruit the B16F10 melanoma-specific T cells in the tumor microenvironment.

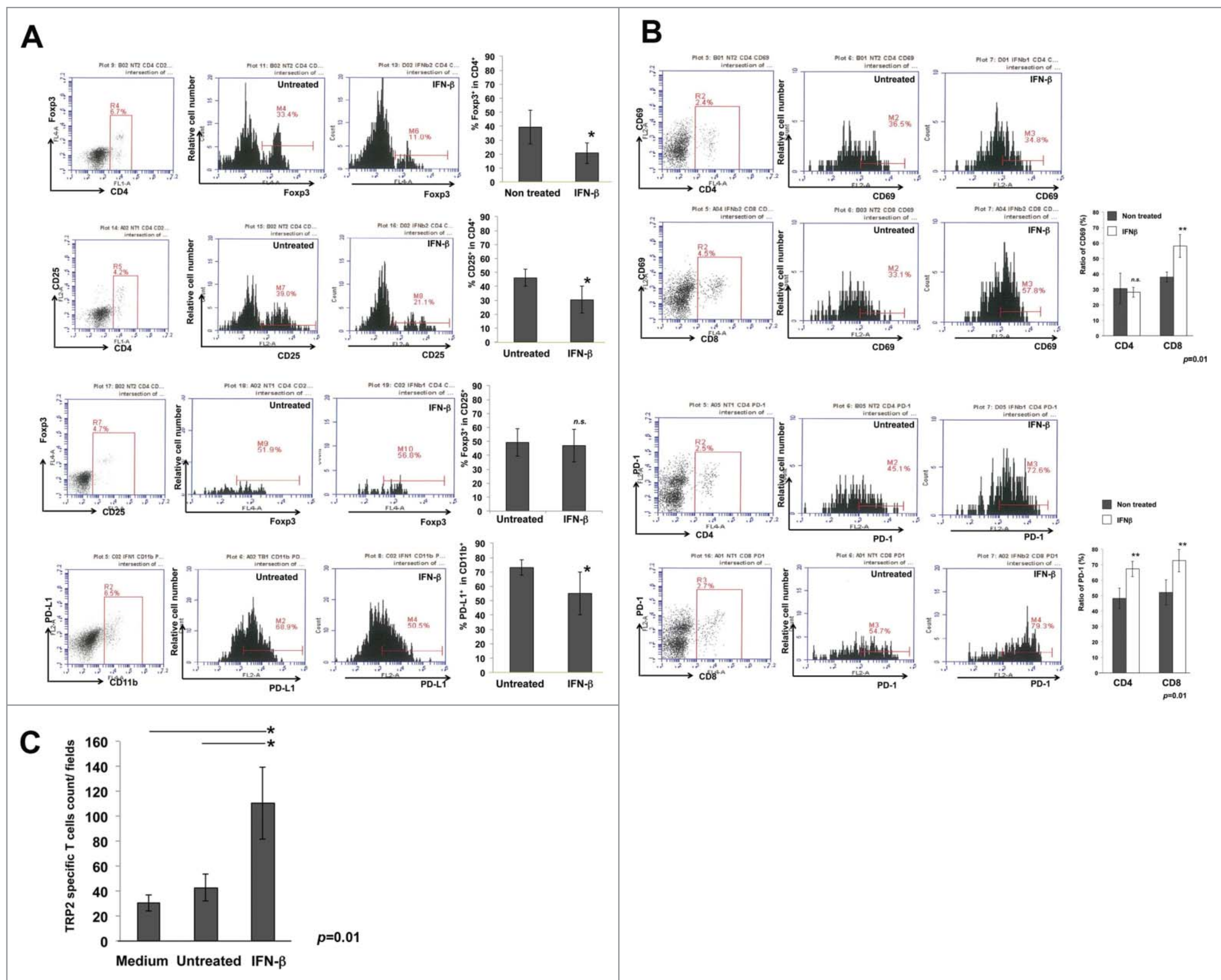

Figure 2. Peritumorally administered IFN-β modifies the profiles of TILs in B16F10 melanoma. Intracellular expression of Foxp3 on $CD4^{+}$ cells (closed square) or $CD25^{+}$ cells, or surface expression of CD25 on $CD4^{+}$ cells or PD-L1 on $CD11b^{+}$ cells in tumors was analyzed by flow cytometry (**A**). Expression of CD69 and PD-1 on $CD4^{+}$ cells or $CD8^{+}$ cells (open square) in tumors was analyzed by flow cytometry (**B**). Tumor size was about 7 mm in diameter. The mean percentage (± standard deviation) of marker-positive cells among $CD4^{+}$ cells, $CD8^{+}$ cells or $CD11b^{+}$ cells obtained in six independent experiments is shown. The chemoattractant activity of culture supernatant of $CD11b^{+}$ cells for TRP2-specific $CD4^{+}$ T cells was evaluated by using a Neuro Probe AP48 chamber, as described in Materials and Methods (**C**). * marks a significant ($P < 0.05$) difference.

IFN-β enhances the antitumor effect of anti-PD-1 Ab against B16F10 melanoma

IFN-β modified the chemokine profiles of TAMs in B16F10 melanoma, decreased the recruitment of Tregs and PD-L1-expressing TAMs, and augmented the recruitment of PD-1-expressing, activated T cells in B16F10 melanoma. Therefore, we hypothesized that IFN-β could enhance the antitumor effects of anti-PD-1 Ab against B16F10 melanoma. We examined the therapeutic effects of IFN-β in combination with anti-PD-1 Ab *in vivo* by using the established B16F10 murine melanoma model.[22,23] We treated B16F10 melanomas (5 mm in diameter) on the backs of mice by intraperitoneal injection of anti-PD-1 Ab (0.25 mg/ mouse) with or without peritumoral injection of IFN-β (10,000 U) three times a week for two weeks. For the control antibody, we used rat IgG (0.25 mg/ mouse). Anti-PD-1 Ab in combination with IFN-β significantly suppressed the growth of B16F10 melanoma (**Figs. 3A, B**). Interestingly, anti-PD-1 Ab treatment with or without IFN-β significantly suppressed the expression of PD-1 on $CD4^+$ and $CD8^+$ cells (**Fig. 3C**).

$CD163^+CD206^+$ macrophages in in-transit melanoma

For the clinical application of the administration of IFN-β with anti-PD-1Ab (nivolumab), we next investigated the immunomodulatory effects of IFN-β on human melanoma. As we and others have previously reported (21, 24, 34), instead of CD11b, the representative markers for human TAMs are CD163 and CD206. Therefore, we first employed immunohistochemical staining for the serial section of in-transit melanoma before and after administration of IFN-β focusing on the profiles of TAMs (CD163, CD206). Our present data showed that the administration of IFN-β increased the numbers of $CD163^+$ macrophages in the lesional skin of in-transit melanoma. Interestingly, there was no significant increase in $CD206^+$ cells (**Fig. 4A**). We determined the number of immunoreactive cells for five patients before and after the administration of IFN-β (**Fig. 4B**).

The immunomodulatory effect of IFN-β on M2 macrophages derived from peripheral blood monocytes

Since peritumoral administration of IFN-β significantly increased the number of $CD163^+$ cells and did not affect the number of CD206 cells in the lesional skin of in-transit melanoma, we investigated the immunomodulatory effect of IFN-β on M2 macrophages derived from peripheral blood monocytes, as previously described.[21,24] Flow cytometry analysis revealed that these *in vitro*-generated M2 macrophages were positive for CD163 and CD206 (**Fig. 5A**). In parallel with lesional skin of in-transit melanoma, the additional IFN-β significantly decreased the expression of CD206 on M2 macrophages (**Fig. 5A**). Next, we evaluated the Th1-related chemokine (CXCL10, CXCL11) and Th2-related chemokine (CCL17, CCL18, CCL22) mRNA expression in M2 macrophages. The mRNA expression of Th2 chemokines was significantly decreased by IFN-β (**Fig. 5B**). In contrast, the expression of Th1 chemokines was significantly augmented by IFN-β (**Fig. 5B**). To

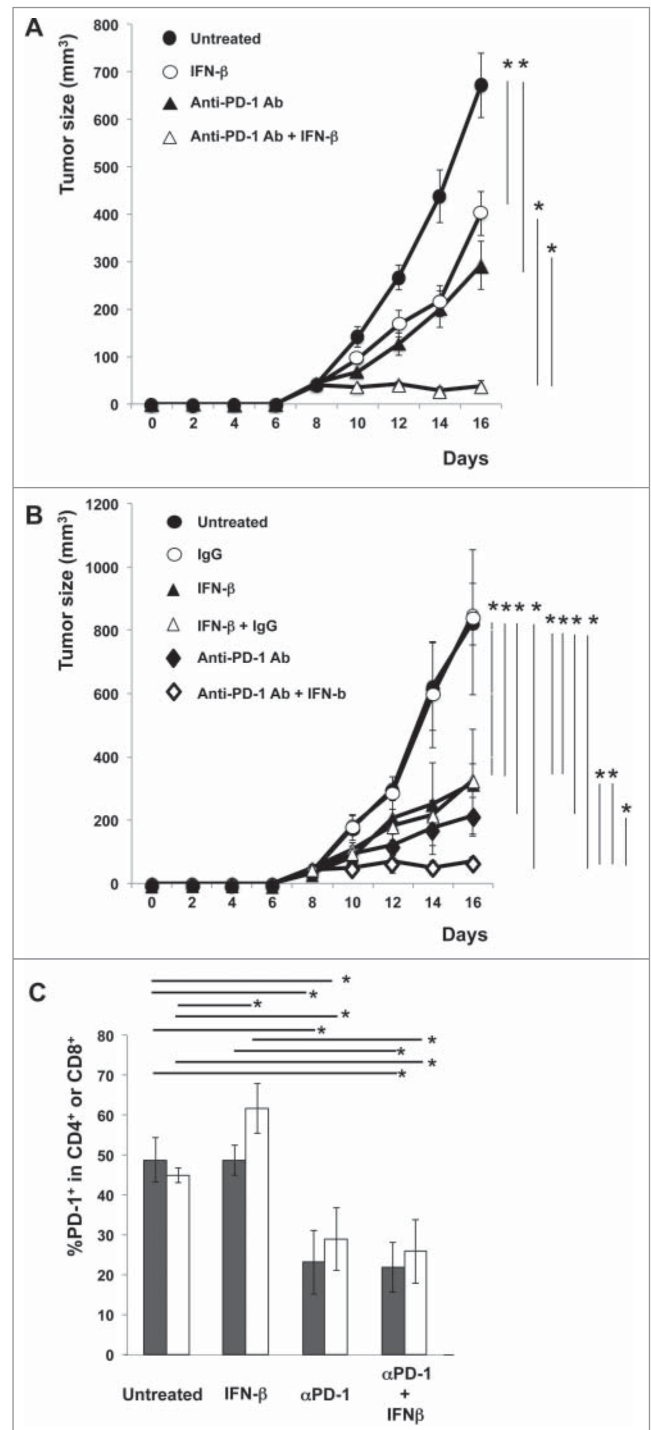

Figure 3. IFN-β in combination with anti-PD-1 Ab suppresses B16F10 melanoma growth. We subcutaneously injected 100 μL of 2×10^6 cells/ mL of B16F10 melanoma cells, and at day 6, we injected IFN-β (10,000 U) intradermally in the peri-lesional area of the tumor with i.p. injection of anti-PD-1 Ab (0.25 mg) (**A**) or control rat IgG (0.25 mg) (**B**). Representative data from one experiment of two independent experiments are shown (**A**: n = 10 for each treated group, **B**, **C**: n = 5 for each treated group). * marks a significant ($P < 0.01$) difference. Expression of CD69 and PD-1 on $CD4^+$ cells (closed square) or $CD8^+$ cells (open square) in tumors was analyzed by flow cytometry after treated with anti-PD-1Ab and/ or IFN-β (C).

further confirm the immunomodulatory effect of IFN-β on the chemokine profiles by M2 macrophages, we performed ELISA (**Fig. 5C**). Consistent with the mRNA expression, the production of Th2 chemokines was significantly decreased by IFN-β, while the production of Th1 chemokines was significantly augmented by IFN-β (**Fig. 5C**).

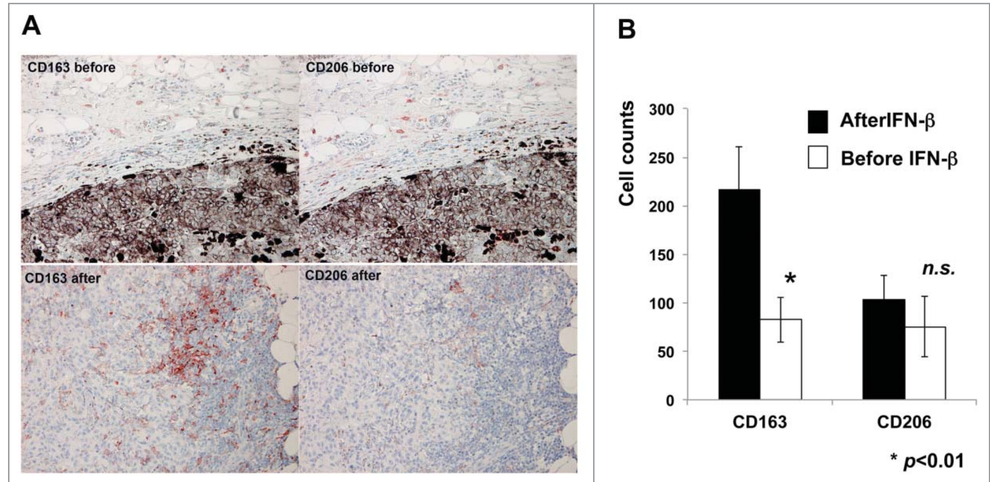

Figure 4. $CD163^+$ cells and $CD206^+$ cells in the lesional skin of in-transit melanoma patients before and after IFN-β treatment. Paraffin-embedded tissue samples from the lesional skin of in-transit melanoma patients were deparaffinized and stained with anti-CD163 Ab or anti-CD206 Ab (**A**). Sections were developed with liquid permanent red (Original magnification ×100). Summary of the numbers of $CD163^+$ cells and $CD206^+$ cells in melanoma samples from five patients before and after the administration of IFN-β. Three representative fields of each section were selected from the dermis associated with dense dermal lymphoid infiltrate. The number of immunoreactive cells was counted by using an ocular grid of 1 cm^2 at a magnification of ×400. The data are expressed as the mean and SD of the number of stained cells. * marks a significant ($P < 0.05$) difference.

$CD4^+Foxp3^+$ Tregs and $PD\text{-}L1^+$ cells in in-transit melanoma

Since *in vitro* analysis suggested a significant effect of IFN-β on M2 macrophages, we further examined the immunomodulatory effect of IFN-β on the tumor microenvironment in patients with in-transit melanoma. In parallel with mouse B16F10 systems and mRNA expression and protein production in human *in vitro* systems, the ratio of $Foxp3^+$ cells on $CD4^+$ cells (**Fig. 6A, B**) was significantly reduced by the administration of IFN-β (**Fig. 6C**). As previously reported,[12] Tregs increase the PD-L1-expressing cells, such as TAMs, in the tumor to maintain the immunosuppressive microenvironment. Therefore, we employed immunohistochemical staining for PD-L1 before or after the administration of IFN-β. In parallel with the decreased ratio of Tregs, the ratio of PD-L1 to CD163 was significantly reduced (**Fig. 6D**) by peritumoral injection of IFN-β in patients with in-transit melanomas.

Discussion

Peritumoral injection of IFN-β occasionally suppresses tumor growth by increasing the number of cytotoxic cells, such as $TIA1^+$ cells and $CD8^+$ cells in the lesional skin of in-transit melanoma,[5] which suggests the potential use of IFN-β for the treatment of metastatic or in-transit melanoma as an immunomodulatory reagent. In contrast to the clinical findings, in a mouse melanoma model, peritumoral administration of IFN-β does not suppress tumor growth *in vivo*.[22,23] This discrepancy may be explained by the immunomodulatory effect of IFN on cancer stroma. For example, IFNγ augments the expression of suppressive molecules, such as PD-L1 and CD271, on the melanoma cells to suppress the activation of melanoma-specific CTLs.[25,26] Notably, the expression of PD-L1 on antigen-presenting cells is indispensable for the induction of inducible (i)Tregs,[27] and PD-L1 enhances and sustains the Foxp3 expression and the suppressive function of iTregs.[27] In contrast, another report indicated that high-dose IFNα2b reduces the circulating Tregs in melanoma patients, which leads to the therapeutic effects of IFNα2b.[28] These reports suggest the possible mechanisms of the immunomodulatory effects of IFN on the tumor microenvironment through Tregs.

Tregs play a pivotal role in maintaining peripheral tolerance that actively suppresses effector T cells.[27] In the tumor site, in concert with TAMs, Tregs maintain the immunosuppressive microenvironment and promote tumor growth.[10,12,27] Indeed, both myeloid-derived suppressor cells, which are known as immunosuppressive immature macrophages, and Tregs determine the prognosis of advanced melanoma patients.[29] In addition, Mahnke et al. reported that the depletion of Tregs in melanoma patients *in vivo* resulted in enhanced immune functions and the substantial development of antigen-specific $CD8^+$ T cells in vaccinated individuals.[30] More recently, Telang et al. assessed the efficacy of depleting Tregs in stage IV melanoma patients and concluded that the depletion of Tregs had a significant clinical effect in unresectable stage IV melanoma patients.[31] These reports suggested that depletion of Tregs could be an optimal supportive therapy for human melanoma.

Tregs modified the phenotype of tumor-infiltrating macrophages to express inhibitory B7-homolog molecules and to produce IL-10 in both human and mouse models.[12,15] As we previously reported, the depletion of Tregs significantly decreased the expression of PD-L1 on myeloid-derived suppressor cells and reduced the tumor growth.[12] Moreover,

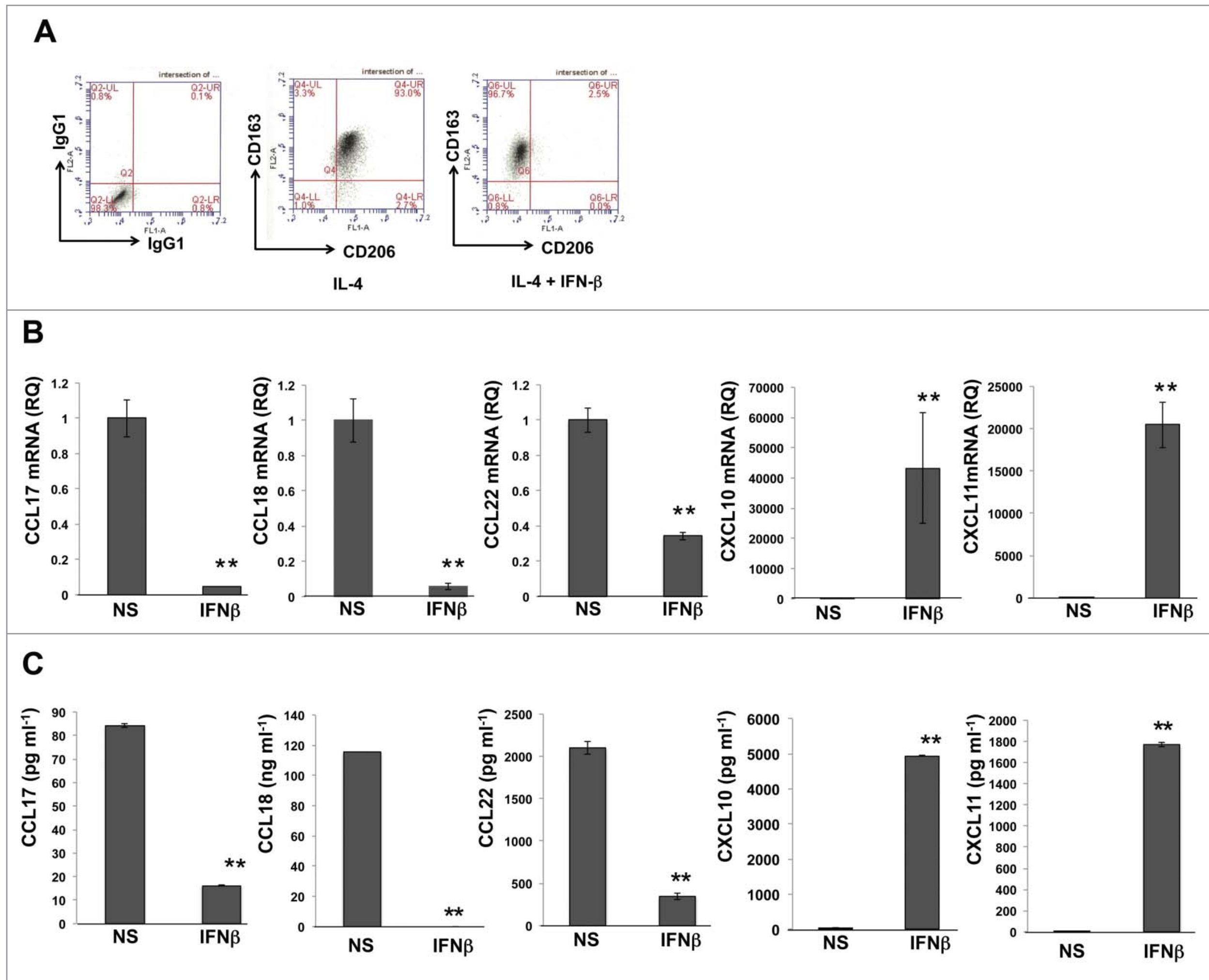

Figure 5. IFN-β induces the expression and production of Th1-related chemokines by M2 macrophages. M-CSF-induced M2 macrophages from peripheral blood mononuclear cells were stimulated by IFN-β. After 48 h of stimulation, the cultured cells were harvested, and the expression of CD163 and CD206 was examined by flow cytometry (**A**). Six hours after stimulation, total RNA was recovered from macrophages and then reverse transcribed into cDNA. Quantitative real-time PCR was conducted to determine the number of copies of cDNA for each chemokine, and the relative mRNA expression levels were calculated for each gene and each time point after normalization against Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by using the ΔΔCt method. The data from each donor were obtained by triplicate assays, and then the mean ± SD was calculated (**B**). The culture supernatants of M2 macrophages stimulated with IFN-β were collected after 48 h of stimulation. Their chemokine production was measured by ELISA (**C**). Representative data from at least three independent experiments are shown. ** marks a significant ($P < 0.01$) difference.

Tiemessen et al. reported that $CD4^+CD25^+Foxp3^+$ Tregs produce IL-10, IL-4, and IL-13 and steer monocyte differentiation toward alternative activated M2 macrophages in humans.[15] Notably, human M2 macrophages produce a substantial amount of Treg-related chemokines such as CCL17 and CCL22.[21,23] Accordingly, we hypothesized that IFN-β might cause M2 macrophages to differentiate into other types of macrophages that produce chemokines related to antitumor Th1 responses. To prove our hypothesis, we used the B16F10 melanoma model and *in vitro*-generated M2 macrophages. As we expected, IFN-β modified the chemokine profiles of macrophages, suppressed the recruitment of Tregs, and increased the activated T cells in the tumor site. Notably, these activated $CD8^+$ T cells co-expressed PD-1, which suggested an impairment of CTL function.[32] Interestingly, the administration of anti-PD-1 Ab strikingly augments the antitumor effects of IFN-β against B16F10 melanoma. In aggregate, our present data suggest a possible supportive protocol for inoperable melanoma with multiple in-transit metastases by using nivolumab and peritumoral injection of IFN-β.

In the present study, we demonstrated the immunomodulatory effects of IFN-β on TAMs to reduce Tregs in the lesional skin of melanomas in both humans and mice. Since IFN-β augments the PD-1 expression on T cells in melanomas, the administered anti-PD-1 Ab is necessary to induce the therapeutic effects of IFN-β. Our present data suggest that IFN-β in combination with anti-PD-1 Ab could be a successful immunotherapy for the treatment of melanoma.

Materials and Methods

Reagents

Recombinant murine IFN-β was provided from Daiichi-Sankyo Pharmaceutical Co, Ltd, Tokyo, Japan. Blocking monoclonal Ab against mouse PD-1 (RMP1-14) was prepared as described previously.[33] We used the following antibodies (Abs) for flow cytometry: rat monoclonal Abs for mouse $CD4^+$ (L3T4) APC, mouse $CD8^+$ (53-6.7) APC, mouse CD11b (M1/70) APC, mouse CD69 (H1.2F3) PE, mouse PD-1 (RMP1-30) PE, mouse PD-L1 (MIH5), and an anti-Foxp3 APC staining kit. We used the following Abs for immunohistochemical staining: mouse monoclonal Abs for human $CD4^+$ (Nichirei, Tokyo, Japan), human CD163 (Novocastra, UK) and CD206 (LifeSpan Bioscience, Seattle, WA USA); and rabbit polyclonal Abs for human Foxp3 Ab (Abcam) and human PD-L1 (ProSci, Poway, CA USA). We also used recombinant human M-CSF and recombinant human IL-4 (Peprotech, London, UK) and human IFN-β (Peprotech, London, UK) for cell culture.

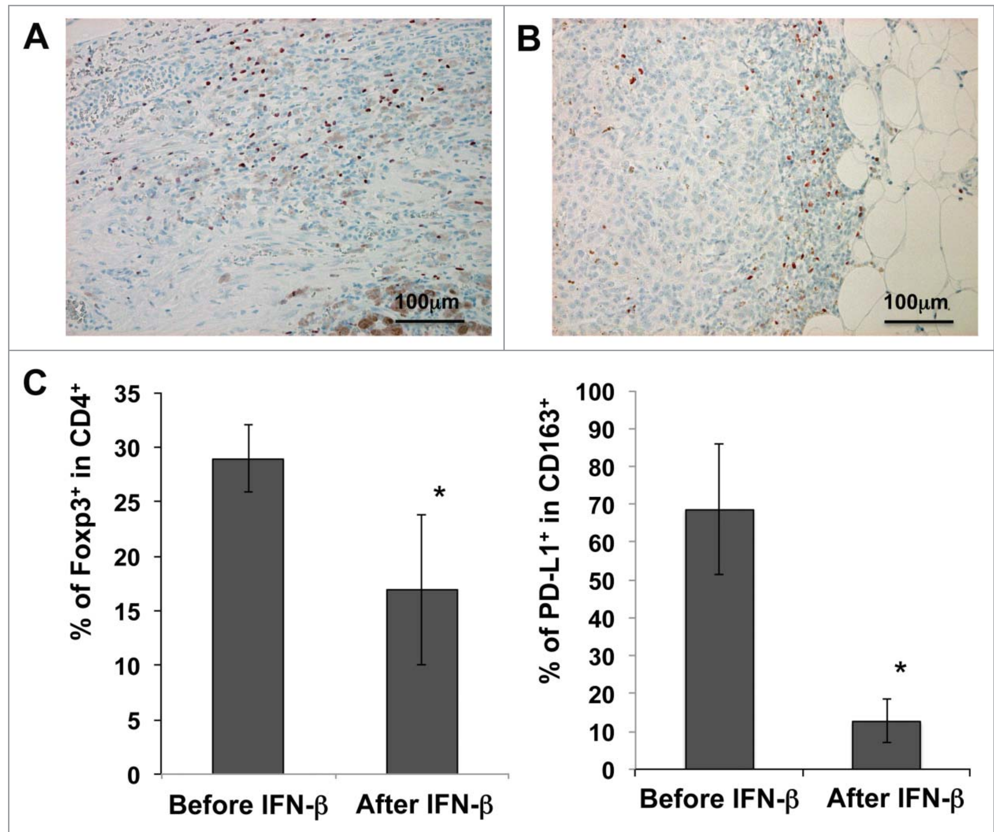

Figure 6. IFN-β modifies the profiles of TILs in the lesional skin of in-transit melanoma patients. Paraffin-embedded tissue samples from the lesional skin of in-transit melanoma patients were deparaffinized and stained with anti-Foxp3 Ab. Sections were developed with liquid permanent red (Original magnification × 100) (**A**). Summary of the ratio of $Foxp3^+$ cells on $CD4^+$ cells and $PD\text{-}L1^+$ cells on $CD163^+$ macrophages in five cases of melanoma samples from five melanoma patients before and after the administration of IFN-β (B). The number of immunoreactive cells was counted by using an ocular grid of 1 cm^2 at a magnification of × 400. The data are expressed as the mean and SD of the number of stained cells in each area. * marks a significant difference ($P < 0.05$), ** marks a significant ($P < 0.01$) difference.

Ethics statement for animal and human experiments

The protocol for the animal study was approved by the ethics committee of Tohoku University Graduate School of Medicine for Animal Experimentation, Sendai, Japan (Permit number: 2013-053). The research complies with the Tohoku University Graduate School of Medicine's Animal Experimentation Ethics guidelines and policies. All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. The protocol for the human study was approved by the ethics committee of Tohoku University Graduate School of Medicine, Sendai, Japan (Permit number: 2013-1-27). All patients provided informed consent.

Animals and melanoma cell line

C57BL/6 mice and BALB/c mice (5 to 8 weeks old) were purchased from Japan Shizuoka Laboratory Animal Center (Shizuoka, Japan) and housed in the animal facility at the Tohoku University Graduate School of Medicine. The murine melanoma cell line, B16F10, was obtained from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's minimal essential medium supplemented with 10% heat-inactivated fetal calf serum (FCS). All mice were bred under specific pathogen-free conditions at the Tohoku University Graduate School of Medicine.

Tumor inoculation and treatment

B16F10 melanoma cells (100 μL of 2 × 10^6 cells/mL) were subcutaneously injected into female C57BL/6 mice as described previously.[22] For qRT-PCR and FACS analysis, 10,000 U recombinant mouse (rm) IFN-β was peritumorally injected on day 7, and the tumor was harvested on day 9. For qRT-PCR, whole tumors were frozen with liquid nitrogen and then crushed with Cryo-Press (Microtec, Chiba, Japan). Total RNA was extracted by using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. For FACS analysis, whole tumor cells were incubated with collagenase 4 and DNase I for 20 min at 37°C, followed by mechanical disruption, as previously described.[12] After single-cell suspensions were obtained, FACS analysis was performed. A total of 1 × 10^5 cells were resuspended

in PBS/1% FCS and incubated with a combination of Abs for 30 min at 4°C. For the detection of Foxp3 expression, we fixed and permeabilized cells by using Cytofix/Cytoperm solution (BD Bioscience) according to the manufacturer's protocol. After washing, cells were kept in PBS/1% FSC. In all assays, cells were analyzed by using a C6 flow cytometer (Acuri Cytometers Inc., Ann Arbor, MI). For the therapeutic experiments, we measured the size of established tumors with a caliper (Mitsutoyo, Utsunomiya, Japan) and estimated their volume by using the formula: $\pi/6 \times$ length $\times$ width.[23] Starting on day 6, we intradermally injected 10,000 U of rmIFN-β at two points in close vicinity of the tumors three times a week. All assays contained five mice per group and were performed at least three times. Tumor-bearing animals were sacrificed when the tumors displayed severe ulceration or reached a size of 1,000 mm^3. For the PD-1 blockade, anti-mouse PD-1 Ab (0.25 mg) was administered intraperitoneally on days 6, 10, 13, and 17 after the tumor inoculation.

RNA extraction and quantitative real-time PCR experiments

Total RNA was extracted by using an RNeasy Micro kit (Qiagen, Courtaboeuf, France) in accordance with the manufacturer's instructions. The RNA was eluted with 14 μL of RNase-free water. DNase I treatment (RNase-Free DNase Set; Qiagen Courtaboeuf, France) was performed to remove contaminating genomic DNA. Reverse transcription was performed with the SuperScript VILO cDNA Synthesis Kit (Invitrogen). Amplification reactions were performed by using an Mx 3000P Real-Time Quantitative PCR System (Stratagene). Relative mRNA expression levels were calculated for each gene and each time point after normalization against GAPDH by using the ΔCt method or ΔΔCt method.

Purification of $CD11b^+$ cells

$CD11b^+$ cells were isolated from tumors by using MACS beads (Miltenyi, Bergisch-Gladbach, Germany), as previously reported.[12] Briefly, tumors were incubated with collagenase 4 and DNase I for 20 min at 37°C, followed by mechanical disruption. After single-cell suspensions were obtained, dead cells were depleted by using a dead cell removal kit from Miltenyi according to the manufacturer's protocol. Thereafter, aliquots of 10^7 cells were incubated for 15 min with 10 μL of anti-CD11b beads (clone M1/70.15.11.5) in 100 μL MACS buffer (PBS, 0.5 mmol EDTA, 0.5% BSA) at 4°C, washed two times with MACS buffer and subjected to two consecutive rounds of separation via magnetic MS columns (Miltenyi). This procedure yielded predominantly $CD11b^+$ cells with purity greater than 80% as assessed by FACS analysis.

Cytokine ELISA

$CD11b^+$ cells from the tumors and spleens of each group were cultured with LPS (0.5 μg/mL) in 96-well flat bottom plates. After 24 h of culture, supernatants were collected. Secretion of CCL22, CCL2, CXCL9, CXCL10, and CXCL11 was determined by ELISA kits (R & D Systems), according to the manufacturer's instructions. For PBMo-derived M2 macrophages, after the 7 d culture, supernatants were collected and the secretion of CCL17, CCL18, CCL22, CXCL10, and CXCL11 was determined by ELISA kits (R & D Systems), according to the manufacturer's instructions.

Generation of TRP-2-specific T cells and chemotaxis assay

C57BL/6 mice were immunized with 10,000 irradiated B16F10 melanoma cells once a week for two weeks. Then, $CD4^+$ cells were positively isolated from the spleen and lymph nodes by using MACS beads (Miltenyi, Bergisch-Gladbach, Germany). This procedure yielded predominantly $CD4^+$ cells with purity greater than 95%. $CD4^+$ cells (5×10^5 cells/ well) were cultured with recombinant TRP-2 (10 μg/mL)-pulsed syngeneic bone marrow-derived dendritic cells and IL-2 (30U/ mL) in 24-well plates at 37°C for 7 d. Then, we harvested $CD4^+$ T cells for the chemotaxis assay.

Chemotaxis assay

$CD11b^+$ cells were isolated from tumors by using MACS beads and cultured with LPS (0.5 μg/mL) in 96-well flat bottom plates, as described above. Then, culture supernatants were collected, and the chemotaxis for TRP-2-specific $CD4^+$ T cells derived from B16F10-immunized mice was evaluated by using a Neuro Probe AP48 chamber (Neuro Probe Inc., Gaithersburg, MA). The supernatants from each sample were placed in the bottom wells of the chamber and covered with a 3-μm filter. The top wells of the chamber were sequentially filled with TRP-2-specific $CD4^+$ T cells (generated as described above) at a concentration of 2×10^6 /mL. The chamber was then incubated at 37°C for 4 h. Following the incubation period, the filter was removed and rinsed, and May–Grunwald–Giemsa staining was performed. The number of migrated TRP-2-specific $CD4^+$ T cells was counted by using an ocular grid of 1 cm^2 at a magnification of ×400. Staining of $CD4^+$ T cells was examined in more than three random, representative fields from each section.

Immunohistochemistry for patients with in-transit melanoma

We collected archival formalin-fixed paraffin-embedded skin specimens from five patients with skin metastasis of malignant melanoma treated in the Department of Dermatology at Tohoku University Graduate School of Medicine. Before the antigen retrieval procedure, 0.3% H_2O_2 in methanol was used to inhibit endogenous peroxidase. The five metastatic malignant melanoma samples before and after the IFN-β treatment were processed for single staining of CD206, CD163, Foxp3, and $CD4^+$ and developed with liquid permanent red (Dako A/S, Glostrop, Denmark).

Culture of M2 macrophages from human peripheral blood monocytes

$CD14^+$ monocytes were isolated from peripheral blood mononuclear cells from healthy donors by using MACS beads (CD14 microbeads, Miltenyi Biotec Inc., Sunnyvale, CA) according to the manufacturer's protocol. $CD14^+$ monocytes (2×10 /mL) were cultured in complete medium containing 100 ng/mL of recombinant human M-CSF for 5 d, as previously reported.[24] On the fifth day, monocyte-derived macrophages were treated with recombinant human IL-4 (20 ng/mL) with or without IFN-β (1000 U/ mL).

Statistical analysis

For single comparisons of two groups, the Student's t test was used. The level of significance was set at $P = 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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